DECLARATION OF ANTONINO CATTANEO UNDER 37 CFR 1.132

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

Applicants:	Antonino Cattaneo et al.	Docket No.:	LLG/2006.01/US
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For :	NON-HUMAN TRANSGENIC ANIMALS FOR THE STUDY OF NEURODEGENERATIVE SYNDROMES		

DECLARATION OF ANTONINO CATTANEO UNDER 37 CFR 1.132

I, Antonino Cattaneo, hereby state and declare:

1. I am a co-inventor of the invention described and claimed in the above-referenced patent application (the "subject application").

2. I am the co-founder of Lay Line Genomics (LLG), located in Rome, Italy, the exclusive licensee of the assignee of the subject application. I presently serve as the company's President and Chief Scientific Officer. Among other appointments, I am a Full Professor of Biophysics at the International School for Advanced Studies (SISSA) in Trieste, Italy, and the co-director of the European Brain Research Institute (EBRI) in Rome. I am also a member of the European Molecular Biology Organization (EMBO).

3. I co-authored the 1996 Cattaneo and Ruberti abstract published in the Society for Neuroscience Abstracts 22(1-3): 753 (Cattaneo et al. 1996). I was personally involved in the research that was reported in this abstract. The transgenic mice disclosed in this abstract are not, in fact, the same as the transgenic mice described and claimed in the subject invention. As explained below, the Family A mice described in Cattaneo et al. 1996 differ both genetically and phenotypically from the transgenic mice described in the subject application.

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4. The transgenic mice described in Cattaneo et al. 1996 were obtained by crossing two single transgenic lines, one expressing the variable light chain (Line A), and the other expressing the variable heavy chain (Line C), of the anti-NGF antibody *α*D11. Although not reported in Cattaneo at al. 1996, both Lines A and C expressed low levels of their respective transgenes. The offspring double transgenic mice, designated "Family A" mice, do not express detectable functional anti-NGF antibody in serum during the first month of age. After 1 month, the levels of antibody become detectable, but remain low, at approximately 10 ng/ml, until approximately 12 months of age, when serum antibody levels reach 50-100 ng/ml. As reported in Cattaneo et al. 1996, the only phenotypic difference observed between Family A mice and wild-type mice (other than the expression of the transgene antibody) is a 30% reduction of neurons in superior cervical ganglia. Otherwise, classical NGF target cells were apparently not affected. Phenotypic analysis shows that Family A mice do not display any of the following phenotypic characteristics present in the transgenic mice of the invention: (a) cholinergic deficit, (b) β-amyloid deposition, or (c) the accumulation of phosphorylated *tau* protein. The corresponding data is presented in Exhibit A hereto.

5. The transgenic mice described and claimed in the subject application were generated as follows. Two single transgenic mouse lines homozygous for the light chain, one expressing the light chain at low levels (Line A) and the other expressing the light chain at high levels (Line B), were generated as described in the subject application. In addition, two single transgenic mouse lines homozygous for the heavy chain, one expressing the heavy chain at low levels (Line C) and the other expressing the heavy chain at high levels (Line D), were also generated as described. In order to generate double transgenic mice expressing both the heavy and light chains of the antibody, the single transgenic parent heavy and light chain lines were intercrossed in different combinations,

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including the combinations indicated below ("low" and "high" referring to the level of expression of the indicated variable gene).

a. F (VK low) X M (VH high) = Family 1
Line A Line D

b. F (VK high) X M (VH low) = Family 2
Line B Line C

c. F (VK high) X M (VH high) = Family 5
Line B Line C

6. Crossing Line A with Line D (Family 1), and Line B with Line C (Family 2), yield double transgenic mice that are viable, fertile and thrive to adulthood. On the contrary, crossing the two high expression lines (Line B with Line D), produced no viable offspring.

7. In carrying out the crosses indicated above, as well as other crosses, my colleagues and I were not looking for or thinking about developing a model for Alzheimer's Disease ("AD"). Our objective was to develop a model to study the effects of NGF on the central nervous system. As we evaluated the phenotypes of Family 1 and Family 2 mice, we began to see phenotypic characteristics resembling those observed in humans afflicted with AD, one characteristic after another. This was completely unexpected and quite surprising. Our initial studies revealed a complex and severe set of deficits closely resembling those seen in human Alzheimer's Disease ("AD"). The extensive studies we have undertaken with this model over the past several years has strengthened and confirmed our initial observations. The model has withstood the test of time. Classical NGF target cells, including sympathetic and sensory neurons, are

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severely affected in the transgenic mice of the invention. In the CNS, basal forebrain and hippocampal cholinergic neurons are not affected in the early postnatal period, whereas they are greatly reduced in adult mice. The mice acquire an age-dependent neurodegenerative pathology including CNS-deposition of amyloid plaques, insoluble and hyperphosphorylated *tau* protein, neurofibrillary tangles in cortical and hippocampal neurons, extensive neuronal loss throughout the cortex, cholinergic deficit in the basal forebrain, and behavioral deficits.

8. The level of anti-NGF antibody expression in Family 1 and Family 2 mice reaches 50-300 ng/ml by postnatal day 45 and remains stable thereafter. The expression levels observed at that time point in the Family A mice of Cattaneo et al. 1996 are substantially lower (see Paragraph 4, above).

9. The two independent transgenic mouse Families 1 and 2, generated by different crosses, share a consistent phenotype. Only the crosses between low expressing light chain parental mice and high expressing heavy chain parental mice (Family 1), or high expressing light chain parental mice and low expressing heavy chain parental mice (Family 2), give rise to the double transgenic heterozygotic mice with the characteristic phenotype, as described and claimed in the subject application. This teaching was <u>not</u> provided in Cattaneo et al. 1996, and could not have been anticipated, but is clearly provided in the subject application (see, for example, page 25, line 25 through page 26, line 2). Neither crossing low expressing single transgene lines together, as was done to generate the transgenic mice reported in Cattaneo et al. 1996, nor crossing high expressing single transgene lines together (disclosed in the subject application), produces the claimed invention.

10. The generation of the transgenic mice of the invention is the result of finding the right combination of expression levels between the heavy and light

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chain genes in the double transgenic mice. Functional anti-NGF antibodies must be assembled from heavy and light chains co-expressed in the same cell, and a sufficient but not "overwhelming" number of cells producing functional antibodies is required for the development of the AD phenotype. We found that high level expression of both variable genes is overwhelming, as this leads to a lethal phenotype early in life. Low level expression of both genes appears to result in too few cells producing functional antibody, and this is evidently not sufficient to drive the progression to the AD phenotype. The subject application discloses that the combination of a parental mouse expressing high levels of either the heavy or light chain, with a parental mouse expressing low levels of the cognate chain, is the combination that leads to the generation of the AD phenotype. Prior to the invention, this could not be predicted. With the benefit of the subject application's teachings, the AD phenotype is now predictable and reproducible.

11. Cattaneo et al. 1996 did not describe the transgenic mouse described and claimed in the subject application. The USPTO indicates, however, that the phenotypic claim limitations not specifically disclosed by Cattaneo et al. 1996 *"are considered to be inherent in the mouse of Cattaneo because its genetic structure is indistinguishable from that of the claimed mouse, and the phenotypic characteristics necessarily flow from the genetic structure".* However, the set of phenotypic characteristics of the transgenic mice of the claimed invention are indeed not present in the transgenic mice disclosed in Cattaneo et al 1996, as indicated in paragraph 4, above. Moreover, while the two transgenes integrated into the mouse of the invention and the mouse of Cattaneo et al. 1996 are presumed to be identical, their physical location within mouse chromosomal DNA is not. Similarly, the number of copies of the transgenes integrated into the mouse genome will differ. These differences in transgene integration translate into different genetic architectures, producing different phenotypes.

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12. In the parental transgenic mice, the levels of transgene expression between lines vary as a consequence of these integration differences. Thus, for example, one obtains a high expressing heavy chain single transgenic mouse in Line D, and a low expressing heavy chain single transgenic mouse in Line C. These expression phenotypes carry forward into the offspring of mice generated from crossing the parental lines. As is well known in the transgenic animal field, the process of integrating a transgene into chromosomal DNA is random, and between one founder and another, results in the integration of transgene DNA into chromatin regions that differ in their levels of activity, thus influencing the expression of the transgene (Overbeek, 1994, in Transgenic Animal Technology, Carl A. Pinkert, Chapter 3, pp. 69-114). The presence of promoters, enhancers and other regulatory factors in the vicinity of (or otherwise influencing) the integrated transgene will also affect the expression of the transgene.

13. Therefore, in the context of transgenic animals, the term "genetic structure" must be interpreted to encompasses the overall genetic architecture. On that basis, the mice of Cattaneo et al. 1996 and the mice of the invention are genetically distinguishable. The genetic differences between the Cattaneo et al. 1996 mice and the mice of the invention are indeed what accounts for their widely divergent phenotypes. Based upon the differences in genetic architecture and phenotype, it can be concluded that the transgenic mice disclosed in Cattaneo et al. 1996 do not inherently possess the phenotype of the claimed invention.

14. The integration of any transgene will result in a variable spectrum of expression levels from one transgenic founder to another – this is well known. Even with a relatively small set of founders, one finds high, medium and low expressing animals regardless of the particular transgene. This variability is, in fact, an asset in making a transgenic mouse of the invention, and is part of what makes the development of the model fully reproducible. The variability in

transgene expression levels in parental mice allows for multiple combinations of high and low expressing single transgene parents. This process of carrying out multiple combinations will lead to the right combination.

15. The subject application demonstrates that multiple crosses, using a relatively small number of parental mice, will produce one or more combinations expressing the characteristic phenotype. In particular, Example 1 (beginning on page 24), describes the generation of four single transgenic parent lines expressing high and low levels of the heavy and light chains of the anti-NGF αD11 antibody, and the successful generation of two independent double transgenic families showing the AD phenotype. Reproducing this example is well within the ordinary level of skill in the art. Analysis of expression levels in the transgenic parent mice is routine, and may be carried out, for example, using PCR or Dot Blot analysis on transgene mRNA obtained from the mice. Once high and low expressing parental lines are identified, crosses between a high expressing heavy or light chain mouse, and a low expressing light or heavy chain mouse, respectively, are undertaken and double transgenic mice identified and screened for the characteristic phenotype, as described in Example 2 (beginning on page 27). Therefore, ample guidance is provided to enable one of skill in the art to generate the required single transgene parental strains and use them to reproduce the double transgenic mice of the invention.

16. Further evidence for the reproducibility of the invention is the fact that the methods disclosed in the application resulted in the successful generation of <u>two</u> distinct double transgene mouse families, <u>each</u> displaying the characteristic phenotype of the claimed invention, despite the fact that the two families were obtained by crossing different pairs of single transgene parents (i.e., <u>both</u> combinations of high/low expressing heavy/light chains). The generation of two independent families with a consistent phenotype rules out chromosomal integration-dependent effects.

17. In view of the potential clinical relevance of some of the described actions of NGF, including its potential role in the development of neurodegenerative disorders, a model allowing the study of the role of NGF in adult animals was very much needed at the time the invention was made. However, prior to the invention, it had been shown that the genetic disruption of NGF in transgenic mice produced a lethal phenotype in the early postnatal period, preventing the analysis of NGF function in adult animals (Crowley et al., 1994, Cell 76: 1001-1011), and no comprehensive transgenic model allowing the study of NGF in adult mice had been developed. The absence of such a model represented a severe limitation in the field.

18. To my knowledge, the transgenic mice of the invention represent the most comprehensive model for AD in the world today. No other existing model simultaneously exhibits the combination of AD phenotypic hallmarks. AD is a progressive neurodegenerative disorder of the central nervous system characterized by the accumulation of abnormally processed proteins in neurofibrillary tangles and senile plaques, principally the β-amyloid protein. Cerebral deposition of β-amyloid is an invariant event in AD, common to familial and sporadic forms of the disease. The model of the invention is the first model comprehensive model of AD, and revealed a number of functions of NGF that were unknown prior to the invention. In addition, use of this model enabled us to conclude that chronic deprivation of NGF in the adult nervous system leads to the formation and deposition of β-amyloid in the model animals, suggesting a direct link between NGF signaling and abnormal processing of amyloid precursor protein.

19. The invention was instrumental in raising the financial capital required for the formation of LLG, and has been responsible for the revenues generated over the past 4 years.

20. LLG currently provides commercial access to the AD11 transgenic mouse model of the invention through a variety of service, licensing and/or collaborative agreements. For example, LLG has granted Johnson & Johnson Pharmaceutical Research & Development, L.L.C. ("J&JPRD", Raritan, New Jersey, USA) a non-exclusive license for the AD11 transgenic mouse model. Under a non-exclusive license agreement, J&JPRD is using the AD11 model in its drug discovery research activities to evaluate the efficacy of a variety of proprietary drug substances to treat cognitive disorders, including Alzheimer's disease. In addition, LLG has also entered into a collaborative agreement with Hofmann-La Roche ("Roche", Basel, Switzerland). Under the terms of this agreement, LLG is providing Roche access to brain tissues from the AD11 transgenic model to perform proteomics analysis aimed at the discovery and assessment of new AD-relevant targets.

21. All statements made herein of my own knowledge are true and all statements made on information and belief are believed to be true, and further, these statements were made with the knowledge that willful false statements and the like so made are punishable by fine or imprisonment, or both, under Section 1001 of Title 18 of the United States Code, and that such willful false statements may jeopardize the validity of the application or any patent issuing thereon.

Antonino Cattaneo

Antonino Cattaneo

05/25/2006

Date

EXHIBIT A

DESCRIPTION OF FIGURES 1-3 HEREIN:

Fig. 1: Neurostereological counts to determine the total number of cholinergic neurons in the basal forebrain (BF) of wild type (WT) mice, AD11 Family 2 and AD Family A mice. AD11 Family 2 mice show a decrease in the number of cholinergic neurons with respect to WT mice (* $P < 0.05$), while AD11 Family A mice do not differ from WT mice and show a statistically significant different from AD11 Family 2 mice. (# $P < 0.05$).

Fig. 2: Counts to determine the number of Abeta clusters and plaques in the hippocampus of WT mice, AD11 Family 2 and AD11 Family A mice. AD11 Family 2 mice show an increased number of plaques with respect to WT mice (* $P < 0.05$), while AD11 Family A mice do not differ from WT mice and show a statistically significant difference from AD11 Family 2 mice (# $P < 0.05$).

Fig. 3: Neurostereological counts to determine the total number of phosphotau neurons in the entorhinal cortex of WT mice, AD11 Family 2 and AD11 Family A mice. AD11 Family 2 mice show an increase in the number of phosphotau-positive neurons with respect to WT mice (* $P < 0.05$), while AD11 Family A mice do not differ from WT mice and show a statistically significant difference from AD11 Family 2 mice (# $P < 0.05$).

Fig. 1

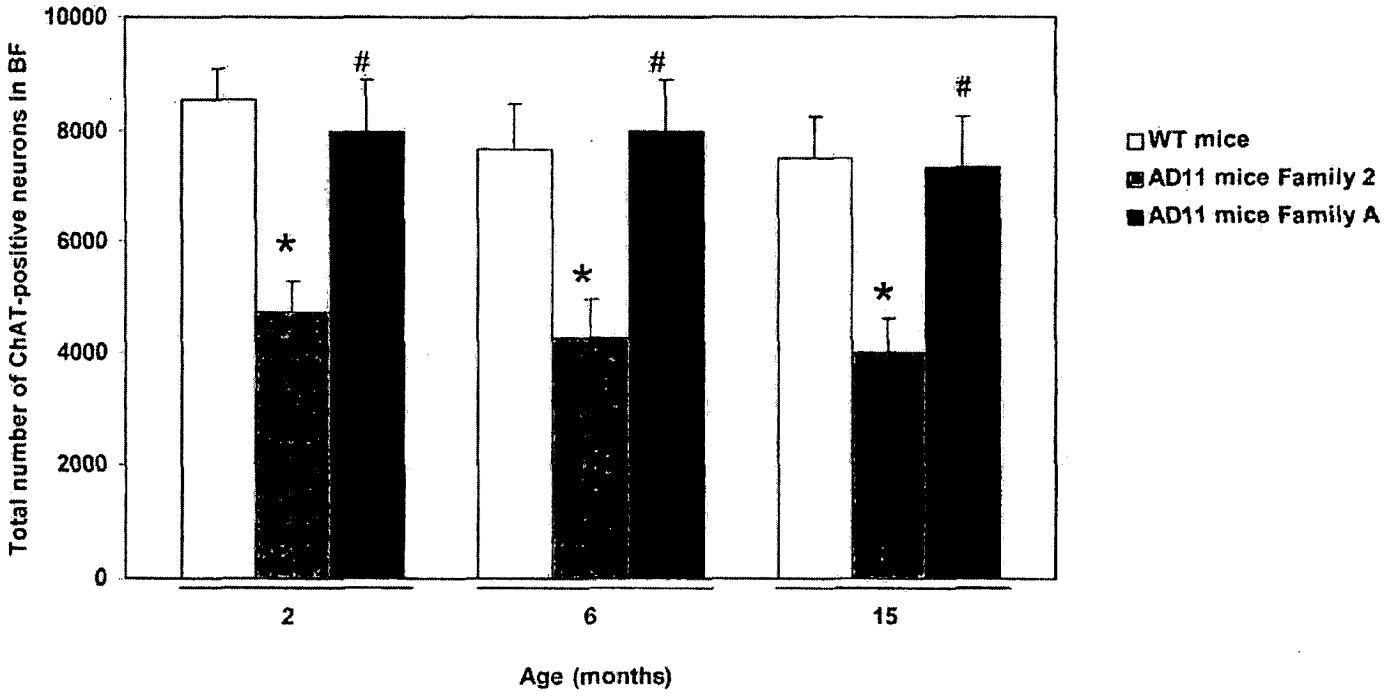

Total number of ChAT-positive neurons in BF
10000
8000
6000
4000
2000
0
2
6
15
Age (months)
*
#
WT mice
AD11 mice Family 2
AD11 mice Family A

Fig. 2

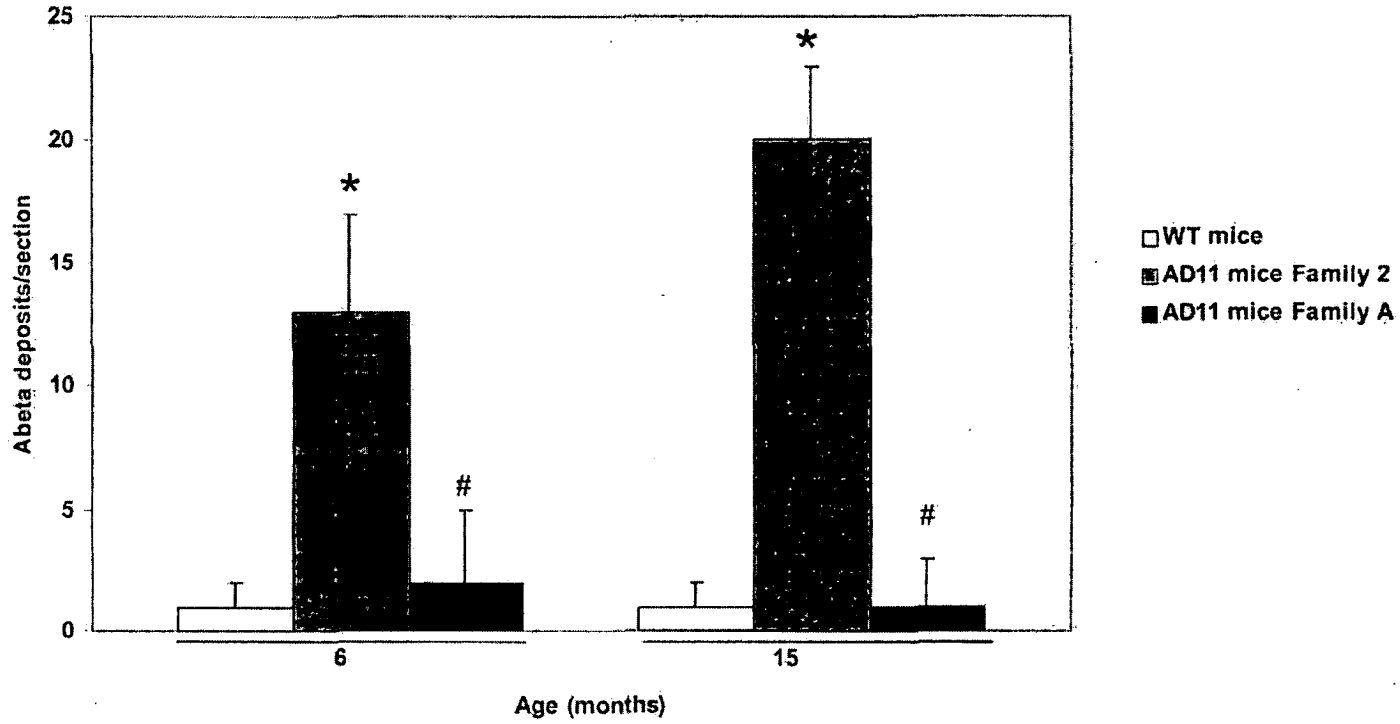
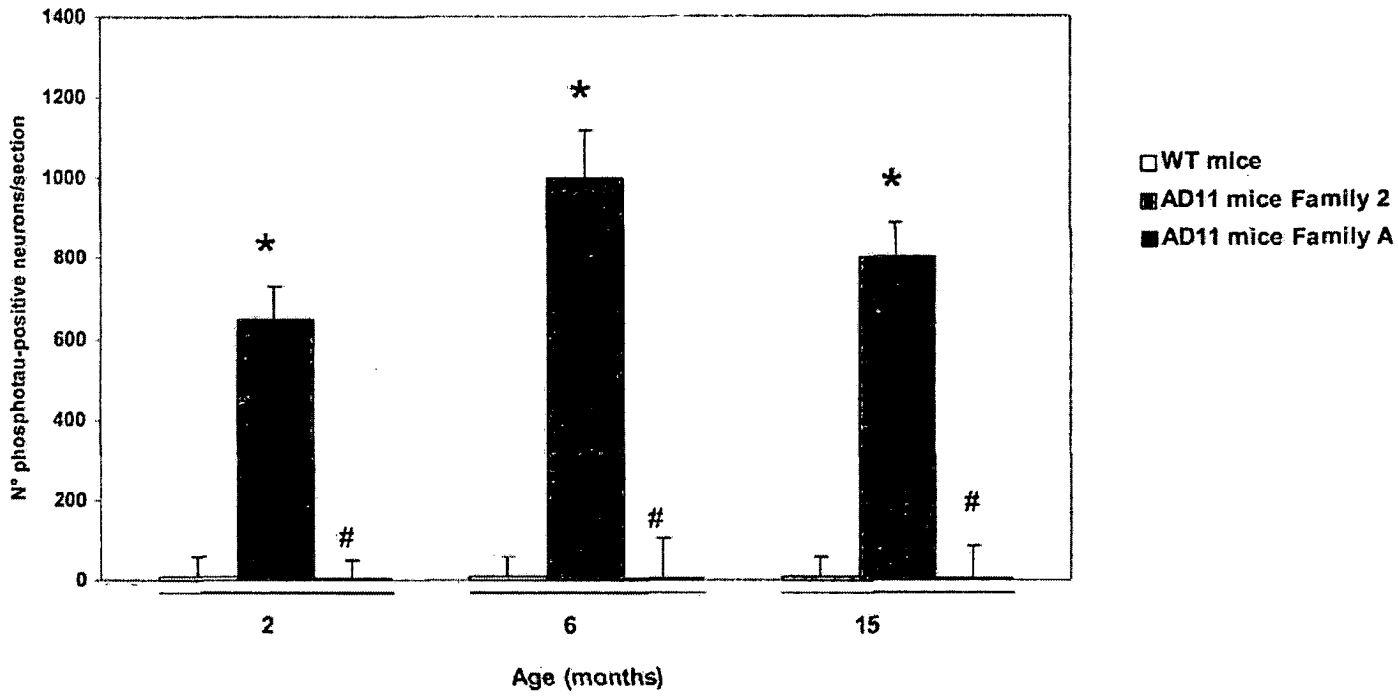

25
20
15
10
5
0
Abeta deposits/section
*
*
#
#
6
15
Age (months)
WT mice
AD11 mice Family 2
AD11 mice Family A

Fig. 3


1400
1200
1000
800
600
400
200
0
N° phosphotau-positive neurons/section
*
*
*
#
#
#
2
6
15
Age (months)
WT mice
AD11 mice Family 2
AD11 mice Family A

TRANSGENIC ANIMAL TECHNOLOGY

A Laboratory Handbook

Edited by

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Front cover photograph: Fertilized mouse ova, from pronuclear zygotes to blastocysts, obtained between 12 and 84 hr after fertilization. Original magnification 100X. Photomicrograph by Carl A. Pinkert.

This book is printed on acid-free paper. ♾

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3

Factors Affecting Transgenic Animal Production

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I. INTRODUCTION

This chapter is intended to provide some general advice about generating transgenic mice. The major goal of the chapter is to help simplify some of the decisions that need to be made when a laboratory begins to do research with transgenic mice. What are good strains of mice to use? Should the transgenic mice be generated by microinjection or retroviral infection, or by genetic manipulation of embryonic stem cells? If microinjection is chosen, what are good vectors to use for the initial training stages? What about record keeping for the mice?

The first half of the chapter provides information about factors to consider when getting started, including husbandry and record keeping suggestions. The second half of the chapter discusses troubleshooting strategies and transgenic phenomenology. Most of the recommendations in this chapter are based on personal experience acquired while running a laboratory that generates transgenic mice by microinjection. The strategies suggested in this chapter have worked well for my laboratory, but they may need to be modified for other research settings. The information in this chapter represents a short introduction to inbred strains of mice, mouse husbandry, mouse breeding, and record keeping. More detailed information about these topics can be found in Hogan *et al.* (1986), Festing (1990, 1992), Hetherington (1987), Otis and Foster (1983), Lang (1983), Rafferty (1970), Green (1975), Dickie (1975), and Les (1975).

II. GETTING STARTED

A. *Microinjection versus Retroviral Infection versus Embryonic Stem Cells*

There are three general techniques for generating transgenic mice: microinjection of one-cell stage embryos, retroviral infection of embryos, and genetic manipulation of embryonic stem (ES) cells. As a general rule, each of these techniques is used for a different purpose: microinjection is used where the major goal is to study expression of new genetic information; retroviral infection (of embryos or ES cells) is used for studies of cell lineage and for random insertional mutagenesis; and ES cells are used for site-directed mutagenesis by homologous recombination.

Microinjection is the most commonly used procedure, mainly because of its reliability. The technique requires a concerted training period (often 3–6 months), but once the protocol is mastered, transgenic mice can be efficiently generated with almost any DNA construction. In most cases, transgenic DNAs introduced by microinjection have been found to be efficiently and reproducibly expressed in trans-

genic mice. Microinjection is the preferred strategy when the objective is to obtain expression of new genetic information (see Chapter 2).

Whereas microinjections are almost always performed on one-cell stage embryos, retroviral infection can be done successfully at various stages of embryonic development, ranging from preimplantation to midgestation. As a result, retroviruses have served as useful markers for cell lineage studies (Soriano and Jaenisch, 1986). For random insertional mutagenesis studies, retroviruses have the valuable attribute of integrating into the host genome without deletions or rearrangements of the chromosomal DNA. One of the best characterized insertional mutations in transgenic mice is the inactivation of the α1 (I) collagen gene by retroviral integration in the *Mov*13 family (Jaenisch *et al.*, 1983; Schnieke *et al.*, 1983; Harbers *et al.*, 1984; Hartung *et al.*, 1986). Retroviruses can also infect ES cells, allowing them to be used for promoter trap and gene trap experiments where there is a preselection for potentially interesting sites of integration (e.g., Friedrich and Soriano, 1991). In contrast to the case with retroviruses, integration of microinjected DNA is often accompanied by major rearrangements or deletions in the host genomic DNA (e.g., Singh *et al.*, 1991; Covarrubias *et al.*, 1986, 1987; reviewed by Meisler, 1992). Such chromosomal changes can complicate the search for the coding sequences of the inactivated gene. Retroviruses have also received attention because of their potential uses for gene therapy (see Chapter 5).

Integration of microinjected DNAs and of retroviruses typically does not occur by homologous recombination, so these procedures are not practical for site-directed mutagenesis. In ES cells, the frequency of integration by homologous recombination is high enough that the targeted integration events can be identified *in vitro* before the cells are used to generate transgenic mice. In those cases where a gene has been cloned, but the phenotype caused by mutation of the gene is not known, ES cells offer a strategy to generate the desired mutants. One technical hurdle to the use of ES cells is mastery of the tissue culture system. The use of ES cells for targeted mutagenesis is described in detail in Chapter 4. Table 1 contains a summary of various features of the techniques for generating transgenic mice.

B. Strains of Mice: Inbred, Outbred, and Hybrid

Inbred strains of mice are defined as strains that have been maintained by successive brother to sister matings over more than 20 generations (Green, 1975). Repetitive inbreeding removes genetic heterogeneity, so that mice of an inbred strain are considered to be genetically identical to each other. There are hundreds of different inbred strains of mice (Festing, 1992). Some of the more commonly used laboratory strains, along with their pigmentation, include the following: A (albino), BALB/c (albino), CBA (agouti), C3H (agouti), C57BL/6 (black), C57BL/10 (black), C57BR (brown), C58 (black), DBA (dilute brown), FVB (albino), NZB (black), NZW (white), SJL (albino), SWR (albino), and 129 (usually albino or chinchilla). The

TABLE 1
Techniques for Generating Transgenic Mice

	Microinjection	Retroviral infection of embryos	Embryonic stem cells
DNA vector	Any cloned DNA, preferably linear with vector sequences removed	Recombinant or wild-type retroviruses	Cloned DNA or retroviruses
Introduction of DNA	Microinjection into pronucleus	Infection after removal of zona pellucida	Electroporation or retroviral infection
Embryonic stage	One-cell stage	Four-cell stage or later	Totipotent ES cells
Embryo transfers	Oviduct	Uterus	Into blastocoel, then into uterus
Genotype of founder mice	Usually nonmosaic	Mosaic	Chimeric
Screening of newborns	Dot blots, Southern blots, or PCR	Southern blots or PCR	Visual coat color markers plus PCR or Southern blots
Copy number of integrated DNA	1–200	1	Can be varied by selection of method for introducing DNA
Percentage of potential founders that are transgenic	10–30%	< 40%	Up to 100%
Expression of the new DNA	Usually	Poor	Enhancer trap, gene trap
Integration	Random; nonhomologous; multicopy, single site	Apparently random using retroviral long terminal repeats (LTRs)	Random plus targeted, depending on method of introducing DNA
Germ line transmission by founders	Usually	Usually	Occasionally a problem
Advantages	a. Straightforward procedure b. Successful expression with many different constructs	Single-copy integration using retroviral LTRs	a. Homologous recombination b. Selection for transformants *in vitro* c. Multiple independent insertions using retroviruses
Disadvantages	a. Physical damage of embryos during microinjections b. Multiple copy integration c. Lack of insertion by homologous recombination	a. Low-level expression b. High titers can be difficult to achieve	Difficult tissue culture system

full name for an inbred strain includes an abbreviation to designate the source of the mice. The abbreviation is placed after a / that follows the name of the inbred strain. For instance A/J mice would be strain A mice from The Jackson Laboratory, whereas FVB/N mice would be FVB mice from the National Institutes of Health. For those strains with a / in the standard name, the abbreviation for the supplier is added to the end of the name (e.g., C57BL/6J mice from The Jackson Laboratory). For more detailed information about naming inbred strains of mice, see Festing (1992).

Fur pigmentation in inbred laboratory mice is controlled primarily by four different genetic loci: agouti (*a*), brown (*b*), albino (*c*), and dilute (*d*) (reviewed by Silvers, 1979). The albino locus encodes tyrosinase, the first enzyme in the pathway to melanin synthesis. When mice are homozygous (*c/c*) for mutations that inactivate the tyrosinase gene, the mice are albino regardless of the genotype at the other loci. All of the common albino strains of mice have the same point mutation in the tyrosinase gene (Yokoyama *et al.*, 1990), indicating that these strains are all derived from a common ancestor. When mice are homozygous or heterozygous for a nonmutated tyrosinase gene (i.e., *C/C* or *C/c*), then the color of pigmentation is determined by the condition of the other genes. If the mice have a wild-type agouti allele (either *A/A* or *A/a*), then the fur will contain both black and yellow bands of pigment and will be agouti (see Fig. 1). If both copies of agouti are mutated (*a/a*), then the hair becomes uniformly pigmented, and the mice are either black (*B/B* or *B/b*) or brown (*b/b*) (Fig. 1). Mice that are homozygous for a mutation in the dilute gene (i.e., *d/d*) show a decreased intensity of pigmentation (not shown).

Hybrid mice are generated by mating mice from two different inbred strains. The mice from such a mating are termed F1 hybrid mice. They are genetically identical to one another but different from either inbred parent. When F1 mice are mated to one another, the offspring are referred to as F2 hybrids. F2 hybrid mice will be genetically different from one another (owing to meiotic recombination and random sorting of the chromosomes) and will contain different mixtures of the genetic variations that were present in the original inbred progenitors. Hybrid mice exhibit a phenomenon termed hybrid vigor. They show enhanced fertility, they respond better to superovulation regimens, and hybrid embryos can be grown efficiently from the one-cell to blastocyst stage *in vitro*. Outbred strains of mice are propagated by nonstandardized matings and therefore retain substantial genetic variability.

Inbred, outbred, and hybrid mice are used for transgenic research. Table 2 contains a short, subjective list of some of the frequently used strains. The inbred strains most commonly used for transgenic research include C57BL/6 (often referred to as "black 6"), FVB, and 129/Sv/Ev mice. The C57BL/6 strain has been used for laboratory research for many years, so that many known mouse mutations are available on the C57BL/6 background. Young C57BL/6 females superovulate well and C57BL/6 blastocysts have been found to be excellent recipients for genetically engineered ES cells (see Chapter 4). FVB embryos are often used for

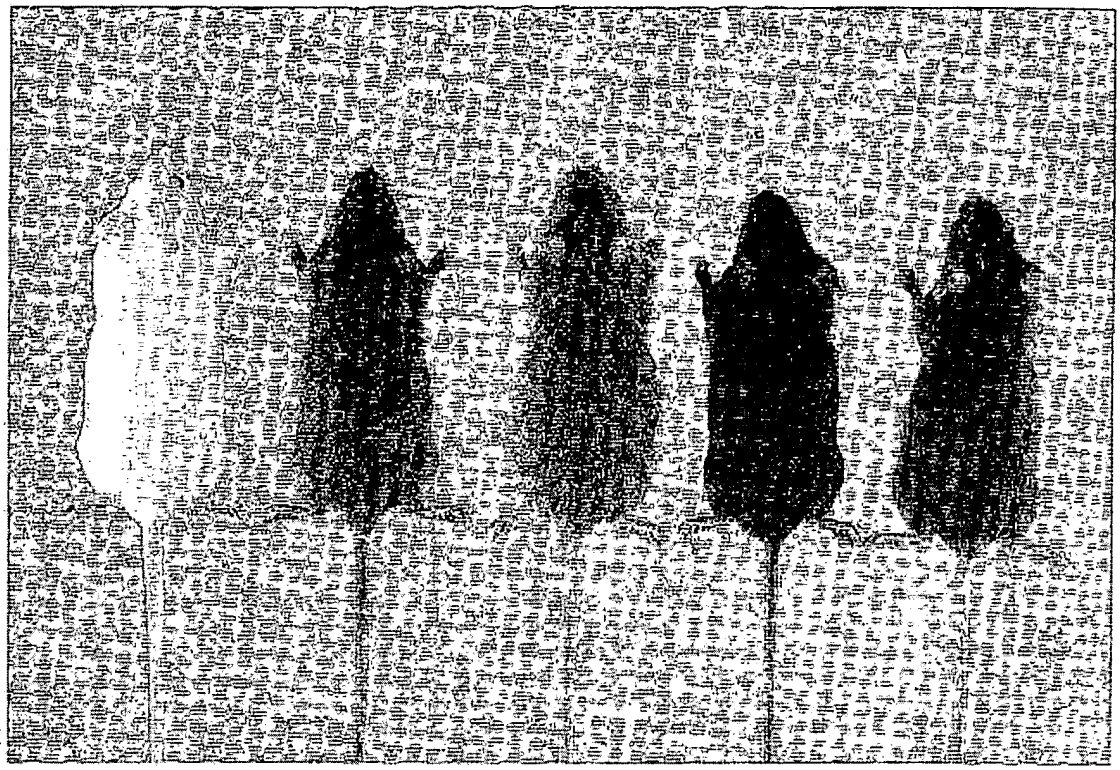

Figure 1. Pigmentation in laboratory mice. Some of the most common pigmentation phenotypes for laboratory mice are pictured. From left to right the mice are albino (genotype c/c), agouti (*A/a B/B C/c*), brown agouti (*A/a b/b C/c*), black (*a/a B/B C/c*), and brown (*a/a b/b C/c*). In agouti mice, the hairs of the fur show a subapical band of yellow melanin (pheomelanin) surrounded by regions of black melanin (eumelanin) (not visible in the photograph). The pheomelanin band is missing in the black and brown mice that are mutated at the agouti locus.

microinjections because they have large distinctive pronuclei (Fig. 2) that are easy targets for microinjection (Taketo *et al.*, 1991). Most of the ES cell lines have been derived from agouti 129/Sv mice. The most commonly used hybrid mice for transgenic research are B6SJL F1, derived by mating the inbred strains C57BL/6 and SJL. Brinster and colleagues (1985) have documented the advantages of the B6SJL mice. Outbred Swiss albino strains such as ICR or Swiss-Webster are often used as recipients for embryo transplantations.

Outbred mice are generally less expensive than inbred or hybrid mice. For example, 6-week-old ICR female mice currently cost less than $2.00 each (Harlan Sprague Dawley, Indianapolis, IN, 1991 catalog), whereas 4- to 5-week-old C57BL/6, FVB, or B6SJL females are approximately $6.00–7.00 each (The Jack-

TABLE 2
Mouse Strains Used for Transgenic Research

Strain	Typical uses
Inbred mice	
C57BL/6	Pronuclear microinjection, recipient blastocysts for embryonic stem (ES) cells
FVB	Pronuclear microinjection
129/Sv	Generate ES cells
Outbred mice	
ICR or CD-1	Pseudopregnant recipient females
Hybrid mice	
B6SJL F1	Pronuclear microinjection
B6D2 F1	Vasectomized males

son Laboratory, Bar Harbor, ME, 1992 catalog; Taconic, Germantown, NY, 1993 catalog). In general, outbred and hybrid mice have better fertility and larger litter sizes than inbred mice. However, the inbred FVB mice have fertility and embryo culture characteristics that nearly match the hybrid and outbred strains (Taketo *et al.*, 1991). The biggest advantage of using inbred mice is the consistency of the

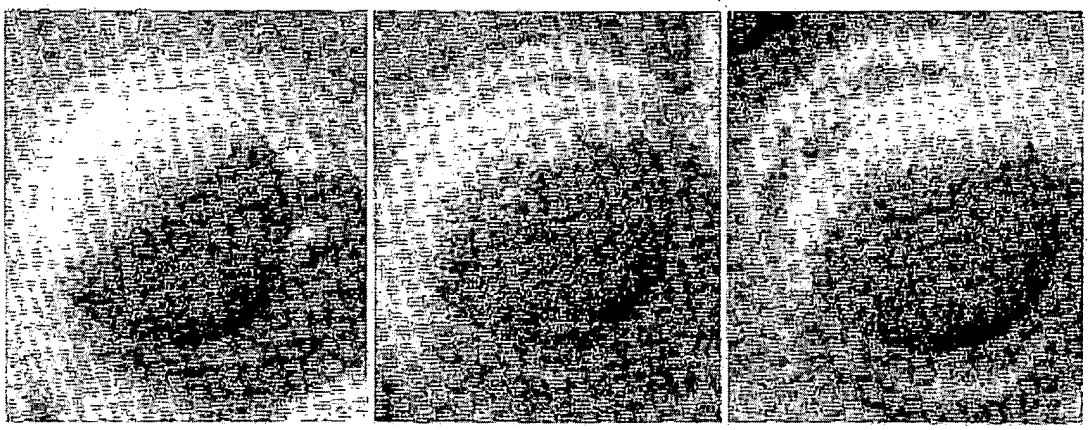

Figure 2. One-cell stage FVB/N embryos. In each embryo, two pronuclei are readily visible. Each pronucleus contains one or more nucleoli (prominent circular organelles within each pronucleus). The two polar bodies are visible for the embryo at left. The FVB/N embryos were cultured in the top of a plastic petri dish (Falcon 1006, Becton Dickinson Labware, Lincoln Park, NJ) and viewed using Hoffman optics. Because the FVB/N pronuclei are large and distinctive, they are easy to inject. Moreover, the fact that FVB/N mice are inbred can simplify the subsequent interpretation of experimental results. [This figure was originally published in Taketo *et al.*, (1991).] (Magnification, 400×.)

genetic background. This can be a pertinent consideration for studies with transgenic mice. When a single DNA construct is used to generate transgenic mice, variability in expression or phenotype between different transgenic mice cannot be attributed to a variable genetic background if an inbred strain of mice is used.

C. Mouse Husbandry: Caging, Mating, Pregnancy, Record Keeping, etc.

1. Animal Welfare

All research with transgenic animals should be carried out as humanely as possible and in accordance with all federal and institutional policies for research with laboratory animals. In the United States, investigators and institutions are expected to comply with the Animal Welfare Act and the Guide for the Care and Use of Laboratory Animals (documents available from the Office for Protection from Research Risks, NIH, Bethesda, MD 20892). As part of this policy, each research institution is required to appoint an Institutional Animal Care and Use Committee, which is required to review and approve animal research proposals and protocols. Before initiating any research with transgenic mice, investigators must document their plans for humane care and research in order to obtain approval from the institutional review committee. In addition, investigators should always confer with the chief veterinarian for the facility where the animals will be housed. The veterinarian will be able to provide information about a variety of important issues, including space allocation for the transgenic mice, acceptable vendors for purchase of mice, responsibilities in the provision of daily and weekly care for the animals, animal husbandry charges, standard housing conditions for the animals, acceptable anesthetics, protocols for animal procedure rooms, treatment of sick animals, disposal of dead animals, and so forth. The chief veterinarian should be consulted concerning all of the husbandry recommendations made in this chapter to be certain that they conform to the standards of the animal facility.

2. Standard Housing Conditions

Laboratory mice are typically housed in polycarbonate cages that are equipped to provide food and water for the mice (see Fig. 3) (Lab Products, Inc., Maywood, NJ). Most facilities use two (or more) standard size cages: a small "shoe box" size of approximately 12 × 8 × 5 in. that can house four or five adult mice, or a nursing female with pups, and a larger cage (19 × 10 × 6 in.) that can house approximately twice as many mice.

Food for the mice is generally provided *ad libitum*. The food is placed either in a metal container that is positioned inside the cage, or alternatively in the metal

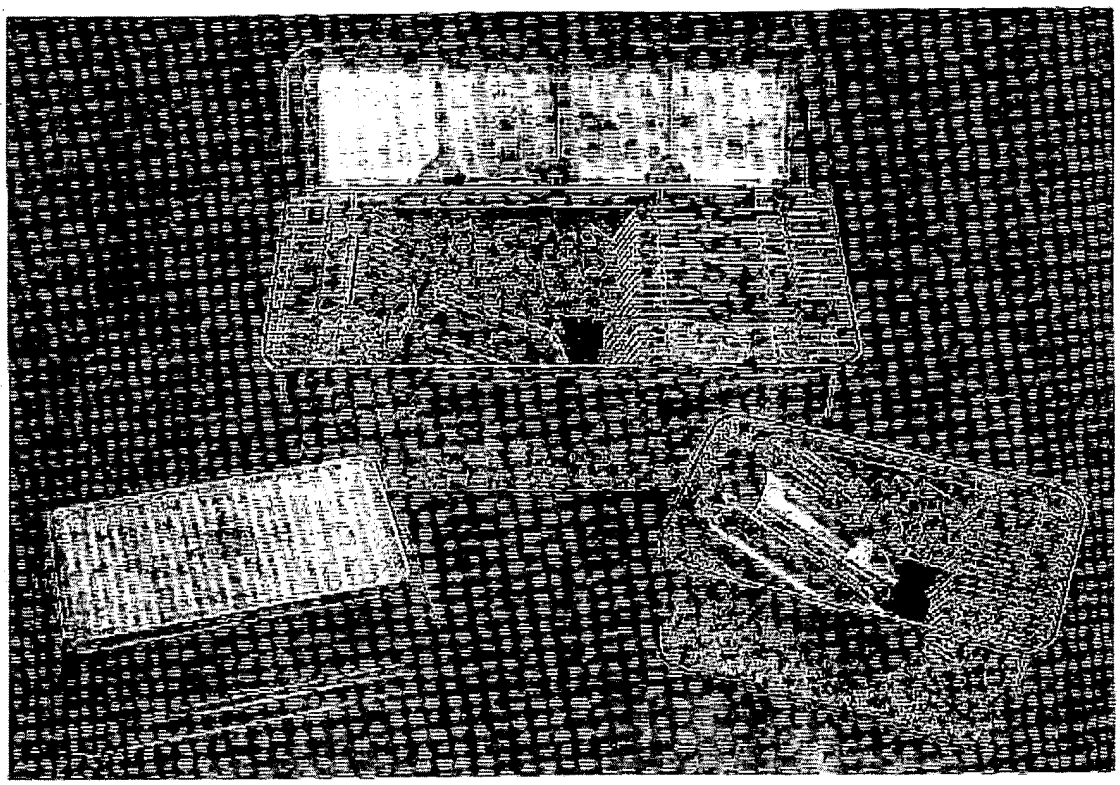

Figure 3. Mouse housing cages. Prototype small and large cages for housing laboratory mice are shown (Lab Products, Inc.). Microisolator tops for each cage size are also pictured. Cage card holders are not shown.

lid on top of the cage. Rodent chow is available in a number of different recipes from suppliers (e.g., Purina Mills, Inc., Richmond, IN). Most animal housing facilities purchase the rodent chow and provide the food as part of the standard animal husbandry services.

Water is also provided *ad libitum*, typically from water bottles equipped with rubber stoppers and sipper tubes (Fig. 3). The water is often acidified to a pH of 2.5 by addition of HCl in order to slow the growth of bacteria in the water. Water bottles should be cleaned and replaced once per week.

Bedding for the mice is traditionally either wood shavings or wood chips and is typically provided by the animal husbandry service. Bedding should be replaced at least once per week. Pregnant females near delivery can be given a cotton block (e.g., Nestlets from Ancare Corporation, North Bellmore, NY) or paper towels to use to build a nest for the pups. Cages with nests should be checked regularly to be certain that the nests do not contact the sipper tubes on the water bottles. Such

contact allows the water to drain from the water bottle, resulting in flooding of the animal cage. Flooded cages can lead to death of young mice by hypothermia or drowning. In general, we do not change the bedding in a cage with a female that is 18 days or more pregnant or is nursing pups that are less than 48 hr old. It is best to leave females relatively undisturbed around the time of delivery.

Special microisolator caging systems have been designed to help prevent the spread of pathogens within a mouse colony. As one example, individual cages can be equipped with special filter tops (Fig. 3) that prevent contamination by airborne debris from other cages in the room. When microisolator tops are used, the bedding should be changed twice a week for cages with more than one mouse, because the cage tops restrict evaporation and air circulation within the cages. An alternative isolation strategy is to use microisolator racks (Lab Products, Inc.). These racks are designed with enclosed shelves and specialized ventilation systems. The shelves are equipped with retractable glass doors that can be opened to allow access to the cages. Microisolator racks are expensive, so they are generally used only for small colonies of mice, or for mice that are housed in special locations.

Housing conditions for mice vary according to the stringency of the procedures used to protect the mice from murine pathogens. The two most common conditions are referred to as "conventional" and "specific pathogen free (spf)" (Lang, 1983; Otis and Foster, 1983). For spf facilities, special precautions are employed to protect the mice from murine pathogens, particularly murine viruses. Personnel working with spf mice are required to follow specific guidelines regarding personal hygiene and clothing when they enter the animal husbandry area. In addition, all experimental supplies and surgical equipment must be decontaminated before they are brought into an spf area. Each spf facility will have its own standard operating procedure, which should be adhered to conscientiously. Conventional housing facilities maintain less stringent hygiene standards, typically allowing mice to be returned to the facility after removal for surgical or experimental procedures.

3. Fertile Females and Embryos for Microinjection

Inbred, outbred, or hybrid one-cell stage embryos can be used for microinjections. To generate inbred embryos, both mating partners must be of the same inbred strain (e.g., FVB × FVB), whereas hybrid embryos can be generated by mating mice of two different inbred strains. Hybrid embryos can also be generated by inbred × hybrid or by hybrid × hybrid matings. If either of the parents in the mating are outbred, the embryos are considered to be outbred.

The two most commonly used inbred strains are C57BL/6 and FVB. Although young C57BL/6 females (3–4 weeks old) superovulate well, often producing 30–40 embryos/female, there are some drawbacks to using the C57BL/6 strain to make transgenic mice, particularly for beginners. C57BL/6 embryos from superovulated females show a high percentage of unfertilized and abnormal embryos

(10–40%), and the pronuclei can be small and difficult to identify. A later drawback is the fact that adult C57BL/6 females are not particularly fertile, with average litter sizes of only 5–6 mice. C57BL/6 females are often poor mothers, so that newborn mortality can occur with a frustratingly high frequency. Both of these characteristics can make it difficult to generate extra offspring within a transgenic family in order to do further research.

In contrast, FVB/N embryos have large, well-defined pronuclei, making them easier to inject (Taketo *et al.*, 1991). Superovulated FVB/N females yield an average of 15–25 embryos/female, they mate with high efficiencies (75–90%), and they produce a high percentage (usually >90%) of fertilized healthy embryos. Moreover, FVB females are excellent mothers, they typically do not cannibalize dead newborns, and the average litter size is 9–10 pups. It should be noted that the FVB strain is not well known outside of transgenic mouse research. The FVB strain, particularly at the immunological loci, has not been as fully characterized as the C57BL/6 strain.

The most commonly used embryos for microinjection are not inbred but are hybrid F2 embryos generated from matings of the inbred strains C57BL/6 and SJL. B6SJL F2 mice have been used for many years by Brinster and colleagues, who found that the hybrid embryos gave a substantially higher frequency of transgenic offspring than inbred C57BL/6 mice (Brinster *et al.*, 1985). The hybrid females superovulate well, and the embryos show the traditional hybrid vigor.

Embryos from outbred strains can also be used to generate transgenic mice. Because outbred mice are less expensive, the novice microinjector should consider using outbred embryos for the initial stages of training. Outbred females superovulate well, and one-cell stage embryos can be maintained in culture to the blastocyst stage in order to monitor percent survival. In contrast to outbred (and hybrid) embryos, embryos from inbred strains allowed to develop *in vitro* often stop development at the 2-cell stage (referred to as the 2-cell block). Embryos from a few inbred strains, such as FVB, will develop efficiently from the one-cell stage to the blastocyst stage in embryo culture medium, such as M16 (Whittingham, 1971; Hogan *et al.*, 1986) or BMOC (Gibco, Grand Island, NY).

Female mice can be induced to superovulate by treatment with hormones. The two main advantages of superovulation are the increase in number of embryos and the synchronization of estrus. Without superovulation, female mice generally release 6–10 oocytes during each estrus. With superovulation the number of oocytes can be increased to as many as 40 per female (Rafferty, 1970; Hogan *et al.*, 1986). Superovulation often works best with younger females that have not yet started their own ovulatory cycle. For many strains of mice, females that are 3–5 weeks of age give the best yield of oocytes on superovulation. The young females will mate successfully, so that this strategy is the standard protocol to obtain embryos for microinjection. The regimen for hormone administration is described in Chapter 2.

In the absence of superovulation, female mice begin to ovulate and become fertile around 5–6 weeks of age. Adult female mice have an estrus cycle of 4–6 days, they are receptive to mating only during estrus, and they cycle regularly

until 7–9 months of age for most strains. Female mice can be housed together in the same cage before mating, during pregnancy, and after newborns have been weaned. In general, pregnant females are placed in separate cages 1 or 2 days before their scheduled delivery. This helps prevent overcrowding, allows unambiguous identification of which pups belong to which mother, and eliminates the possibility that the pups from one mother will be attacked by another female in the cage.

In most research facilities, mice are housed in rooms with no external lighting. A timer-controlled lighting system is used to provide a consistent daily lights-on, lights-off cycle, which is typically 14 hr of lighting, then 10 hr of darkness (for example, lights-on at 6:00 A.M., lights-off at 8:00 P.M.).

4. Fertile (Stud) Males

Male mice typically become fertile at 7–8 weeks of age. When males are housed together from weaning age, multiple males can generally be kept together in the same cage indefinitely. However, experienced males should not be housed together, since they will fight with one another, often until the most aggressive male has killed the other males in the cage. When males are ready to be used for matings, they should be placed in individual cages. In general it helps to give each male his own cage at least 24 hr before setting up a mating in order to give the male time to establish the cage as his territory. Matings are initiated by adding one or more females to each male cage.

We typically use each fertile male for only one or two matings per week. A more frequent schedule leads to lower mating percentages and a substantially decreased percent fertilization. Vasectomized males can be mated more frequently, since their ability to produce adequate sperm is not relevant. However, vasectomized males only occasionally mate more than twice a week, even if given the opportunity.

The fertility of the stud males typically begins to decline when the mice reach 9–10 months of age, at which time they should be replaced with younger males. Male mice remain fertile longer than female mice. In most strains of mice, experienced males remain fertile up to 14–18 months of age, and inexperienced males can often be successfully mated up to 10–12 months of age. Males that look sick or that have stopped mating should be replaced.

The genetic background of the fertile males should be matched to that of the superovulated females. If the females are outbred, then the males can be outbred. If the females are inbred, then the males should be from the same strain in order to maintain the inbred background. Hybrid females are generally mated to either F1 or F2 hybrid males of the same genetic background.

We maintain a cage card with a mating record for each stud male. The mating record indicates whether a plug was found for each date when the male was set up for mating with a superovulated female. Males that do not produce plugs for

four consecutive mating opportunities as well as males that mate less than 50% of the time are replaced. Overly aggressive males will fight with and injure females that are added to their cage. Males that show such behavior more than once are euthanized.

5. Vasectomized Males

The genetic background of the vasectomized males is usually not critical, since these males should not produce any direct descendents. To help recognize the occasional occurrence of an inadequate vasectomy (i.e., a fertile vasectomized male), coat color markers can be used. The vasectomized males can be selected so that inappropriate offspring would have a different coat color than the offspring from injected embryos (e.g., albino versus pigmented).

Vasectomized males should mate consistently and over a reasonable time span. Our favorite males are hybrid B6D2 F1 mice (generated by mating C57BL/6 to DBA/2 mice). The mice are inexpensive and readily available from commercial breeders. The males mate consistently up to at least 1 year of age, and they leave readily identifiable plugs. The males are docile and easy to work with. Males can be purchased already vasectomized (e.g., from Taconic).

6. Recipient Females

Once embryos have been genetically manipulated *in vitro*, they need to be transferred to pseudopregnant recipient females. Because the purpose of the recipient females is to carry the embryos to term and to nurse the newborns to weaning age (3 weeks), it is important to use females that are good mothers. The females should also be relatively docile so that they will allow inspection of the pups, and will accept pups from another mother. Outbred ICR females are inexpensive and make good recipient females.

One strategy to obtain pseudopregnant females is to set up random matings, by placing one or two females per cage with vasectomized males. In a nonsynchronized population, 10–20% of the females will be in estrus on an average day, so that the total number of females to set up for matings should be 5 to 10 times the number that will be needed for the embryo transfers. Mated females are identified by inspection for copulation plugs (Rafferty, 1970). If embryo manipulations are done on consecutive days during the week, this system has the advantage that a large number of females can be set up on the first day, and on subsequent days only previously unmated females need to be checked for plugs.

An alternative strategy to obtain pseudopregnant recipient females is to set up new matings each day using females that are in estrus. Such females can be identified by inspection of the vagina [for swelling, redness, and a rippled folding (wrinkling) of the vaginal wall; see Hogan *et al.* (1986)]. When the females are pre-

screened for estrus; 50% or more of the females should be successfully mated overnight. The advantage of prescreening is that fewer females need to be checked for plugs each morning. The disadvantage is that new females need to be set up for matings every evening.

A third strategy to obtain pseudopregnant females is to perform superovulations to induce estrus in the recipient females, then to mate the females to vasectomized males. In our experience, naturally mated females have a substantially higher rate of pregnancy after embryo transfer than superovulated females, so we use superovulation only as a last resort.

Pseudopregnant females that are not used for embryo transfers will typically return to an estrous cycle 8–11 days after mating. The females can be saved and remated if desired.

7. Pregnancy

The gestational period in the laboratory mouse is 19–20 days. Females that are more than 11–12 days pregnant with a normal size litter of 7–10 pups can be recognized by visual inspection for abdominal enlargement.

As stated earlier, pregnant recipient females are generally given individual clean cages 1 or 2 days before delivery. If the females are provided with nesting materials such as paper towels or crushed cotton squares, they will generally build a nest in which to place the newborns after delivery. Females that do not build a nest often turn out to be poor mothers. Pregnant females that are housed with a male can be left with the male during delivery if additional offspring are desired. Female mice go into estrus postpartum and are receptive to mating the night after delivery. As a result, female mice can be nursing one litter and pregnant with a second litter at the same time. The gestational time for the second litter will often be extended to 21–25 days, as a consequence of delayed implantation (Hogan *et al.*, 1986). The delayed implantation allows the first set of pups to be weaned at 21 days of age before the second set of newborns arrive.

Female mice often cannibalize fetuses that are dead at birth, so if one wishes to look for prenatal or perinatal lethality of transgenic embryos, then a cesarean section (C-section) should be performed prior to the scheduled delivery. In those cases where one wishes to deliver live pups by C-section, the delivery should be done 24 hr before the scheduled normal delivery, that is, at 18.5 days postfertilization. C-sections are also advisable for females that are pregnant with only 1–3 fetuses, since these fetuses can become oversized, impeding normal delivery. We have not had much success performing C-sections where we keep the pregnant female alive to nurse the newborn pups. The surgical trauma appears to disrupt nursing behavior and milk production. Therefore, it is essential to have a "foster" mother to nurse the C-section pups if they are to be kept alive. The foster mother should have delivered her own litter within the previous 48 hr, and ideally the nam-

ral pups of the foster mother should have a different coat color from the C-section pups so that they can be visually distinguished. In addition, the foster mother should have pups that are well fed, with milk visible in their stomachs.

The protocol used for the recovery of midgestation embryos (Hogan *et al.*, 1986) can also be used for C-section delivery of live fetuses. The first step is to sacrifice the pregnant female by cervical dislocation, then to open up the peritoneal cavity and externalize both horns of the uterus. By visual inspection, one can count the number of full-grown fetuses and also identify instances of postimplantation embryonic lethality. Embryos that die shortly after implantation can be recognized by the presence of degenerated (brownish) decidua. (Decidua are sites of cellular proliferation within the uterus induced by fetal implantation.) Fetuses that die at later stages can be identified by their small size and inappropriate color.

To deliver the live fetuses, use a pair of scissors to make an incision at the vaginal end of each uterine horn, and advance the scissors along the antimesometrial side of the uterus up to the oviduct in order to expose the interior of each uterus. With a little practice, this procedure can be done so that each fetus remains encased within its own fetal yolk sac. To deliver each fetus, the individual yolk sacs can be grasped with a pair of watchmaker's forceps and opened by incision with scissors. The edges of the yolk sac can then be peeled back around the fetus, so that the umbilical cord can be located and severed with the scissors. Each fetus can be cleaned up by rolling it back and forth on a paper towel, then transferred to a paper towel on top of a 37°C slide warmer. The entire litter of newborns should be delivered as quickly as possible. Once the pups have been delivered, gently stimulate each fetus every 15–30 sec to induce breathing. Forceps can be used to gently pinch the skin behind the neck, or to spread the legs apart, or to simply roll the newborns on the paper towel. Healthy fetuses will begin to make sporadic inhalations within 1–2 min of delivery, particularly on stimulation. Over the next 1–2 min the fetuses should begin to breathe more regularly. Once breathing becomes stable, the fetuses should become a healthy pinkish color as the blood becomes oxygenated. Fetuses that survive the C-section delivery should be allowed to stabilize, then transferred to a foster mother.

Our strategy for this transfer is to begin by removing the foster female from her cage and placing her on the cage top. Next a sufficient number of the natural pups are removed so that when the C-section newborns are added to the cage the total litter size will be 5–8 pups. The foster mother is more likely to accept the new pups if some of her own pups are left in the cage. The C-section newborns are transferred to the recipient cage and placed with the remaining newborns of the foster female. The entire collection of pups is then buried under approximately 5 mm of the bedding that is already present in the cage of the foster female. This is done to transfer the odors of the cage to the C-section pups before the foster female is returned to the cage. After the appropriate information is entered on the cage card, the foster female is returned inside her cage. One additional recommendation is to treat the nose of the foster female with an alcohol swab just prior to placing her back in her

cage. The alcohol treatment will cause a short-term loss of olfactory discrimination. The behavior of the foster female should be monitored for the first few minutes after she is placed back in the cage. In most instances, the foster female will move around the inside of the cage sniffing at both the cage top and the bedding, as well as digging in the bedding at various locations in the cage. The pups in the cage will emerge from the bedding within 2–3 min if the female does not directly dig them out. When the foster female accepts the new pups, the female will begin to groom herself and the new pups. On the other hand, when the female does not accept the new pups, the usual response is to pick up the pups at the nape of the neck and to carry them rapidly around the inside of the cage as if looking for some way to dispose of them. If the newborns are not accepted by the first fosrer female, then it is typically necessary to repeat the procedure with a new foster mother.

If the natural pups cannot be distinguished from the C-section pups by coat color, then it may be helpful to physically mark one set of pups for later identification. One strategy is to use scissors to snip off a small toe on one of the feet. A small amount of bleeding accompanies toe removal, but the bleeding quickly stops and does not cause the foster female to reject the marked pups. The marked pups can be identified at weaning age by simple visual inspection for missing toes.

Milk production in lactating females is stimulated by nursing, and very small litters often do not provide adequate stimulation to maintain milk production. If the recipient females from 1 day's worth of injections all have small litters, it is often beneficial to consolidate the pups so that the litters average 6–7 pups.

8. Newborn Mice

New litters of mice should be monitored within 12–24 hr after birth to check for newborns that have died, and also to be certain that the newborns are being properly cared for and nursed. At the time of delivery, the mother will eat the placenta and fetal yolk sac associated with each newborn mouse. In addition, the mother will typically groom the newborns by licking in order to stimulate breathing. Within 2–4 hr after birth, properly fed newborns will have milk in their stomachs, which can be seen through their skin. If the mice are not being fed 12 hr after birth, or if they are found unattended and scattered around the cage, they should be transferred to a foster mother as described in the previous section.

Mice are typically old enough to wean by 21 to 23 days after birth. If the mother is not pregnant with another litter of pups, we often leave the pups in with the mother until 25 days of age. If the mother is pregnant with another litter, the first litter should be removed promptly at 21 days of age. If the older pups are not removed, newborn mice in the next litter will be trampled and will not survive. Adolescent mice should weigh 9–10 g or more at weaning. Mice that are smaller than this should be left with the mother, since they often have trouble surviving on their own. At weaning, the mice are sorted by sex and placed into new cages.

9. Mating of Transgenic Mice

Founder transgenic mice can be mated to nontransgenic partners to generate additional transgenic mice. Transgenic offspring of such matings will be hemizygous for the transmitted integration site(s). Interbreeding of mice from different transgenic families that carry the same construct is generally not done. Matings (intercrosses) between hemizygous mice within a given family can be set up in order to generate mice that are homozygous for a transgenic insert. In order to identify homozygous transgenic mice, quantitative Southern or dot-blot hybridizations are generally required. Homozygosity can be confirmed by mating (crossing) the mice to nontransgenic partners. All of the offspring will be transgenic if one of the parents was homozygous. Homozygous mice are often examined in detail for the possibility of insertional mutations. In some families, viable homozygotes may be absent due to a mutation that causes embryonic or perinatal lethality. When the homozygotes of both sexes are viable and fertile, they can be used for subsequent matings (incrossing) to maintain the transgenic family without the need for DNA screenings at each generation.

10. Record Keeping

There is no standardized record keeping system for transgenic research of which I am aware. A good record keeping system should be able to provide updated information about every mouse in the research colony. For each mouse it is important to know whether the mouse is transgenic, what DNA vector the mouse carries, who the parents and the offspring of the mouse are, when the mouse was born, and how the mouse has been characterized to date. I describe below some features of the record keeping systems that we use.

a. Identification Numbers for Each Mouse. Mice that belong to any one of the following three categories are assigned individual, unique identification numbers: recipient females, potential founder mice, or offspring within a transgenic family. Each category is allocated a series of numbers which can be preceded by a letter (e.g., R for recipient females, F for potential founders, and T for transgenic families). Within each category, new mice are assigned sequentially increasing numbers. (One person in the laboratory is responsible for maintaining log books that are used to allocate the identification numbers.) In conjunction, we use a homologous set of ear tags that have the identification numbers engraved on them (National Brand and Tag Company, Newport, KY). When a potential transgenic mouse reaches weaning age and is anesthetized to obtain tissue for DNA isolation, the appropriate ear tag is affixed. Ear tags are applied to recipient females at the time of embryo transplantation. Once the ear tags have been applied, individual

mice are uniquely identified and can be readily located within the colony. For studies of embryonic development in transgenic families, the embryos are assigned identification numbers, but from a series for which no corresponding ear tags are purchased.

A common alternative system for keeping records on transgenic mice is to keep track of mice within individual transgenic families. Each mating pair is assigned an identification number, and offspring are identified by ear punching (Hogan *et al.*, 1986). Ear punching can be combined with toe clipping to mark up to 10,000 different mice. This system is less expensive than using ear tags, but mistakes in mouse husbandry are easier to correct if the mice have ear tags.

b. Transgenic Family Identification Numbers Investigators traditionally assign some type of family identification number (or letter) to each transgenic founder mouse and to all of its offspring. Most laboratories follow their own conventions in the assignment of these identifiers. However, the Committee on Transgenic Nomenclature of the Institute of Laboratory Animal Resources (ILAR) has adopted specific guidelines for naming transgenic families of mice. These guidelines are given in the Appendix at the end of this chapter. At a minimum, a laboratory-assigned number and laboratory identifier (three letters) should be given to each transgenic family. In this fashion each transgenic family becomes uniquely identified. The nomenclature rules should be adhered to by all transgenic research laboratories.

c. Cage Cards Mouse cages are generally equipped with cage card holders for 3 × 5 in. index cards. Each card should carry information about the mice inside the cage. We use two different formats for the information: one for cages with mice that are all of the same sex and a different one for mating pairs or nursing females (Fig. 4). For unisex cages the following information is written on each cage card: sex of the mice, number of mice, identification numbers for each of the mice, date of birth, date of weaning (or date of receipt if the mice were received from a vendor), identification numbers for the parents, DNA vector, identification number of the transgenic family, information about recognizable phenotypes for any of the mice, and information about the results of screening the mice for the transgenic DNA (see Fig. 4). When a cage contains a mating pair, comparable information is entered on the cage card: identification numbers for the mating mice, date of birth of the mating pair, identification numbers of the parents of the mating pair, DNA vector, identification number of the transgenic family, date of birth and number of pups on delivery (including the number of stillborn mice), identification numbers assigned to the newborn mice, and comments about phenotypes of the newborn mice (see Fig. 4).

To maintain some organization within the mouse housing area, mice that have similar genetic backgrounds are housed in proximity to each other. When more than one room is available to house the mice, each room can be allocated to different sets of mice. For example, one room could be specifically for the matings to gen-

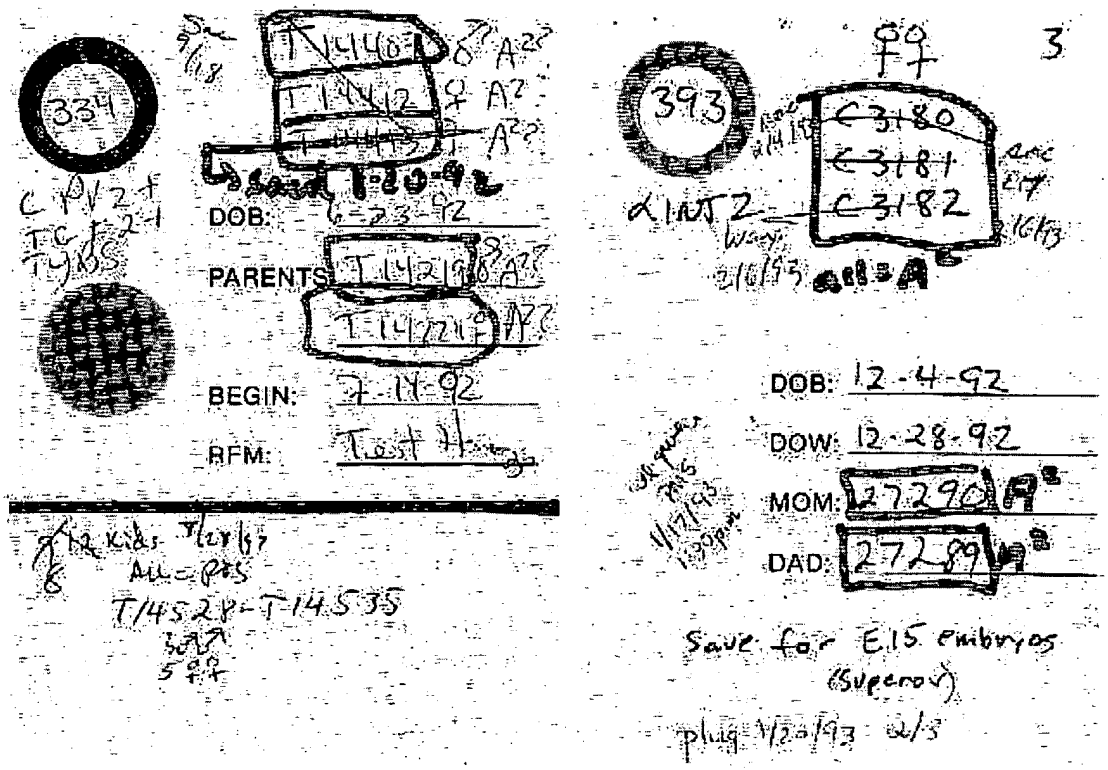

Figure 4. Cage cards. Information about mice within a cage is typically entered on a 3 × 5 in. index card that is held in a cage card holder on each cage. Prototype cage cards are shown for a mating pair (left) and a cage that contains only mice of the same sex (right). The identification numbers and sexes are entered at the top of each card, along with information about the date of birth (DOB), date of weaning (DOW), and parents. For mating pairs, the start date for the mating is entered (BEGIN), along with information about the reason for mating (RFM). When the offspring are born, the relevant information is recorded, including number of pups and date of birth. New identification numbers are assigned to the offspring and entered on the cage card. At weaning, the sexes of the offspring are recorded. To simplify the recognition of specific sets of related transgenic mice, each construction that is used for microinjection is assigned a code of one small colored dot within a different colored larger dot. These dots are adhesive and are placed in the upper left-hand corner of each card. Each founder mouse is assigned a family identification number, and the family number is written within the smaller dot (334 for the left card, 393 for the right card). The names of the transgenic constructions are written on the cards under the dots. When a mouse is sacrificed, a line is drawn through the corresponding identification number.

erate embryos for injection and pseudopregnant females; a separate room could be used to house transgenic families. To simplify the recognition of various sets of mice we have found that different color index cards are helpful. For example, we use one color card to identify FVB/N mice, a different color for outbred ICR mice,

a third color for vasectomized B6D2 F1 males, and a fourth color for all of the cages that contain potential transgenic mice. In addition, we use a color-coding scheme to identify families of transgenic mice that were all derived from injections with the same DNA vector. Colored dots are added to each transgenic cage card (see Fig. 4). By placing a small dot of one color inside a larger dot of a different color, unique color patterns are generated and assigned to each microinjected DNA. When a founder transgenic mouse is identified, it is assigned a transgenic family identification number, and that number is written on the small dot on each cage of that family. We have found that this cage card identification system greatly simplifies the search for specific cages of mice within an animal housing room. When the mice in a specific cage have all been removed, the cage card is saved and placed in a file (organized by family number) so that it can be retrieved if needed.

d. Pedigree Charts For each family of transgenic mice, a continuously updated pedigree chart is maintained (Fig. 5). Males are designated by squares, females by circles, and mice that die or are sacrificed without sexing are indicated by diamonds. Transgenic mice are indicated by placing a diagonal line within their symbol. Putative homozygous mice are given an X within their symbol. For families where the founder mouse has more than one site of integration, the different sites are designated A, B, C, etc. (which is different from the nomenclature rules in the Appendix), and the relevant site is written below the symbol for each transgenic mouse. Matings are indicated by entering a short descending line below the symbol for each mouse of the mating pair, then by drawing a horizontal mating line to connect the two mice. Offspring are indicated by drawing a vertical line running down from the mating pair line to the next tier of the pedigree chart, followed by a horizontal line on which to indicate information about the offspring (see Fig. 5). Such information will include the date of birth, the identification numbers, and the sexes of the mice, once determined. Mice that are deceased are indicated by drawing an X through the stem line that attaches to their sex symbol (see Fig. 5). The pedigree charts are left in the mouse housing rooms and are updated whenever new information is available. These continuously updated pedigree charts provide a simple summary of the status of each transgenic family.

e. Newborn Records Separate hard copy records about each litter of mice can also be maintained. Desirable information might include number of mice in the litter, identification number and sex of each mouse, parents, date of birth, date of weaning, transgenic vector, transgenic family identification number, phenotype of each mouse, results of genomic screening for each mouse, results of all assays for transgene expression, mating record for each mouse, and information about the date of death, cause of death, and status of any tissues that were saved for each animal. Investigators setting up a new transgenic laboratory will need to decide what type(s) of record keeping system to establish, and then to establish the discipline necessary to maintain the records.

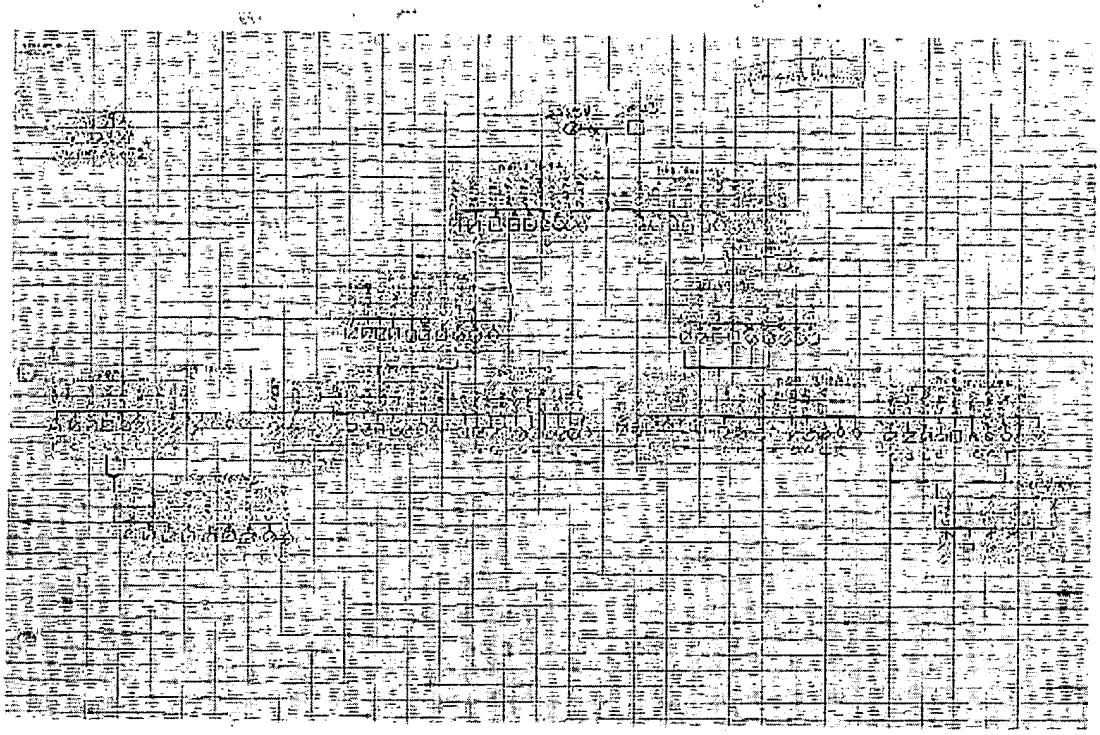

Figure 5. Pedigree chart. Pedigree charts are kept in the room where the mice are housed and are updated whenever new information becomes available. The charts provide a quick overview of the status and history of each transgenic family. Males are indicated by squares, females by circles, and mice that die (or are sacrificed) before sexing are indicated by diamonds. Identification numbers and dates of birth are written above the sex information for each mouse. Transgenic mice are indicated by placing a / within the sex symbol, and information about the phenotype or screening results is entered below the symbol (e.g., c for cataracts). Homozygous mice can be signified by placing an X inside the sex symbol. When mice are sacrificed, we indicate this by drawing an X through the vertical line that attaches the sex symbol to the horizontal litter line.

f. Computerized Record Keeping In some laboratories, it may be preferable to employ a computerized record keeping system, although such systems have not yet achieved widespread use. Ideally, a data management program could be designed so that the relevant information for each cage of mice could be entered into the computer, and the computer would print out an appropriately labeled cage card. The computer could automatically assign identification numbers and could maintain the pedigree charts and newborn records for all of the transgenic families in the colony. Data would need to be entered into the computer for each recipient female.

at the time of embryo transfer, for each litter at the time of birth and also at the time of weaning, and for each mating pair. In addition, data concerning the results of assays on the mice and dates of sacrifice would need to be entered. For such a system to be useful, the computer should be fully portable so that it can be brought into the mouse housing rooms whenever husbandry is performed. I am unaware of any commercially available software programs that are designed for record keeping in a transgenic mouse colony. Custom designed programs for mouse record keeping do exist (e.g., Silver, 1987; or Dr. Richard Woychik, Oak Ridge National Laboratory, personal communication, 1993).

III. TROUBLESHOOTING

A. Assaying for Successful Pronuclear Microinjection

One problem with learning to do microinjections is that it can be a long wait between the time the microinjections are done and the time that the results are known, particularly if one waits until the microinjected embryos have developed into weaning age mice before screening. By the time tail DNA is isolated and screened, it will be nearly 2 months after the time of the microinjections. If a novice is making a consistent mistake, a considerable amount of time and effort will be wasted.

There are a number of constructions that are particularly useful when learning to do microinjections. At the outset, either the metallothionein–β-galactosidase (MT–βgal) construct (Stevens *et al.*, 1989) or alcohol dehydrogenase under control of the Rous sarcoma virus promoter (RSV–ADH) (Nielsen and Pedersen, 1991) can be used to determine whether the microinjections are properly introducing genetic information into the pronucleus. After correct microinjection, either MT–βgal or RSV–ADH can be expressed in embryos at the 2- to 4-cell stage. As a result, embryos can be microinjected, incubated at 37°C for 24–48 hr, then stained for β-galactosidase or ADH activity (Stevens *et al.*, 1989; Neilsen and Pedersen, 1991). When the microinjections are done properly, a significant proportion of the embryos will show histochemical staining.

B. Visual Identification of Transgenic Mice

1. Elastase–*ras*

The promoter for the elastase gene has been shown to be active in pancreatic acinar cells in transgenic mice (Swift *et al.*, 1984; Ornitz *et al.*, 1985). Transgenic mice that carry the elastase promoter linked to the *ras* oncogene develop pancreatic

tumors (Quaife *et al.*, 1987). These tumors are dominant and dramatic and can typically be recognized by simple visual inspection of the perinatal fetus. In a non-transgenic fetus, the whitish pancreas can be identified through the slightly transparent skin. Transgenic fetuses exhibit substantially enlarged pancreata (Quaife *et al.*, 1987; Pinkert, 1990). Pancreatic neoplasia can be confirmed by surgery to allow visual inspection of the viscera. For the novice microinjector, elastase–*ras* permits rapid identification of successful generation of transgenic mice within 3 weeks after injection of the DNA (Pinkert, 1990). One of the advantages of this construct is the fact that it can be used with embryos of any genotype (in contrast to the tyrosinase minigene, which is described in the next section). One drawback to the elastase–*ras* construct is the fact that the pancreatic tumors are generally lethal at an early age for the transgenic mice (Quaife *et al.*, 1987).

2. Tyrosinase Minigene

Classic albino strains of mice have a mutation in the gene encoding tyrosinase, the first enzyme in the pathway to melanin synthesis (Yokoyama *et al.*, 1990). Microinjection of a tyrosinase minigene into embryos of an albino strain of mice can result in gene cure of the albino defect and the synthesis of pigment (Tanaka *et al.*, 1990; Beermann *et al.*, 1990; Yokoyama *et al.*, 1990). The conversion from albinism to pigmentation is easy to recognize (Fig. 6), so the tyrosinase minigene

[redacted]

expensive outbred albino strains such as ICR can be used. Pigmented mice have dark eyes that can be identified by simple visual inspection at birth. In fact, the pigment epithelial cells of the retina begin to synthesize melanin by day 12 of embryonic development (Theiler, 1989), so that transgenic mice can typically be identified by visual inspection of the fetuses 2 weeks after microinjection (Fig. 7). Another advantage of the tyrosinase minigene is the fact that it is not detrimental to the health of the transgenic mice. The tyrosinase minigene is not useful in strains of mice that are already pigmented.

C. Superovulation Problems

1. Poor or No Superovulation

After an overnight mating opportunity, 70% or more of superovulated females should have mated, on average. If the mating percentage is consistently below 50%, either the males or the females may be guilty. A good first step to take in diagnosing the problem is to check for oocytes in the oviducts of the females with no visible plug.

Figure 6. Mice transgenic for a tyrosinase minigene. A nontransgenic FVB/N albino mouse is shown at left. The other three mice represent independent sites of integration (i.e., independent founder mice) for the tyrosine minigene *TyBS* (Yokoyama *et al.*, 1990). The mouse at right is mosaic. [This figure was originally published in a Dutch book entitled *De DNA-Makers, Architecten van het leven* (H. Schellekens, ed.), p. 121, Natuur and Techniek, Maastrich, Netherlands, 1993.]

If the nonplugged females have ovulated a normal number of oocytes (10–20/side) and if the oocytes are mostly fertilized, then the problem is not in the superovulation regimen, but in the identification of copulation plugs. It may help to check for plugs earlier in the morning, before they have a chance to fall out. If the nonplugged females have ovulated, but the eggs are unfertilized, the fault may lie with the males rather than the females. Check to be sure that the males are not too old or mating too often. If the males are not the problem, then it may be the mating conditions. Check to be sure that the lights are set for the proper lights-on, lights-off cycle, and that the lights are truly turning off at night. Also check for problems with loud noises or other disturbances, and be certain that the temperature in the animal room is not elevated (80°F is too hot).

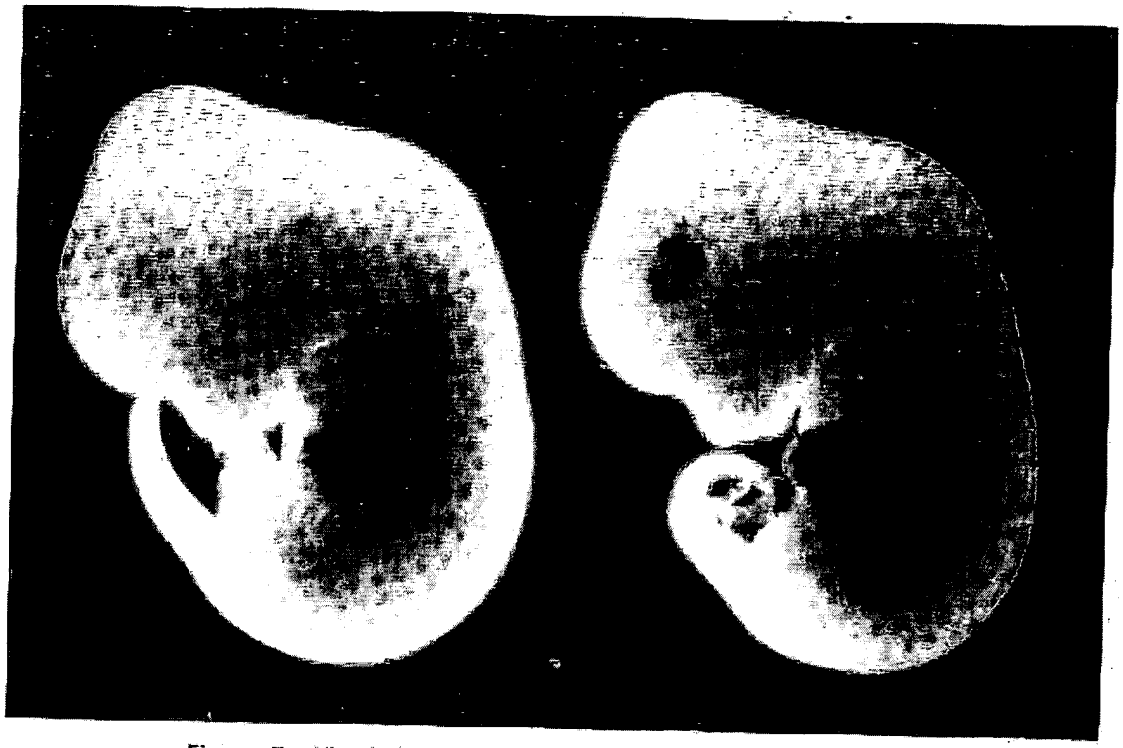

Figure 7. Visual identification of transgenic embryos. Most mice that are transgenic for the tyrosinase minigene can be identified by visual inspection for ocular pigmentation anytime after embryonic day 11 (E11). A nontransgenic E12 embryo is shown at left, whereas a littermate embryo transgenic for the tyrosinase minigene is shown at right. (Magnification, 10×.)

When the nonplugged females have not ovulated, the most likely explanation is a problem with the hormones, particularly the follicle stimulating hormone (FSH), which is usually provided by administration of pregnant mare's serum gonadotropin. If the stock solutions of hormones (50 units/ml) are more than 2 months old, make up new solutions and be sure to store them at 4°C. If the solutions were just recently prepared, check to be certain that they were prepared at the correct concentration. Another possible cause of problems is superovulation of female mice immediately after arrival in a new housing room. A minimum of 48 hr is often required for acclimation.

If the mating percentage is fine, but there are very few embryos, it might be useful to isolate the embryos earlier in the day. In some cases, the cumulus cells disaggregate early and the embryos proceed down the oviduct, making them more difficult to identify and to isolate. The most exclusive test of superovulation

[redacted] persist, try another strain of mice, or obtain the mice from a different vendor.

2. Unfertilized Eggs

If the females have copulation plugs, but the embryos are unfertilized, the problem lies with the males. In most cases, poor male fertility is caused by overuse [redacted]

3. Malformed Eggs

The frequency of malformed eggs is generally a function of the strain of mice and the age of the females at superovulation. In our experience, 10–25% of the eggs from superovulated 4- to 5 week-old C57BL/6 females are immature or malformed, whereas analogous FVB females typically yield less then 5% defective eggs. The younger the females are at the time of superovulation, the higher the percentage of misshapen oocytes. If the percentage of defective eggs is over 25%, then the concentrations of the hormones used for superovulation should be rechecked. Poor health or abnormal stress of the superovulated females can also result in a high percentage of malformed eggs.

When almost all of the embryos appear unhealthy or abnormal, even the fertilized embryos, the most common cause is a problem with the osmolarity or pH of the mouse embryo medium. Alternatively, the CO_2 concentration in the 37°C incubator may be incorrect (it should be 5%, v/v), or the embryos may have been outside of the incubator for too long during embryo isolation or manipulation. (See Chapter 2 for information about embryo culture media and culture conditions.)

D. Colony Problems

1. Males Not Mating

Males not mating is a rare problem, most often caused by overbreeding of the males. If the females are in estrus, healthy adult males will mate at least 75% of the time.

2. No Recipient Females in Estrus

When female mice are housed together, they will often begin to cycle in synchrony. When this happens, there will be days with multiple recipient females in

estrus and other days with very few recipients in estrus. One way to circumvent this problem is to superovulate recipient females when needed. The drawback to this strategy, as stated earlier, is that superovulated females seem to have a poor pregnancy rate when used as recipients. An alternative strategy is to maintain a larger stock of potential recipient females in the colony.

3. High Rates of Abortion in Recipients

Recipients that have as few as one healthy fetus will normally maintain pregnancy to full term. Pregnant females rarely miscarry or deliver before term (<1% of the time). Frequent miscarriage indicates that the females are either sick or unduly stressed.

4. Small Litter Sizes in Recipient Females

When recipient females consistently have small litters (only one or two pups), the problem is unlikely to be simply embryonic lethality of transgenic fetuses, and it is more likely to be a technical problem. The mouse embryo medium might be at fault, the microinjection procedure might be flawed, the concentration (or quality) of the microinjected DNA(s) might be incorrect, or there may be problems with the embryo transfer procedure. To identify the problem, a first step is to do embryo transfers using uninjected embryos. Approximately 75–100% of uninjected embryos should yield healthy newborns. If the percentage is substantially less than that, there is a serious problem with either the culture conditions or the embryo manipulation procedures. A second troubleshooting procedure is to culture injected and uninjected embryos overnight. Most (>90%) of the uninjected embryos should reach the 2-cell stage after 24 hr in culture. If the embryos do not divide, it suggests that there are serious deficiencies in the culture conditions. For embryos that survive microinjection, a majority (50–60%) should divide to the 2-cell stage after overnight culture. If not, either the microinjection procedure is causing irreparable damage, or the DNA solution is lethal. To test the microinjection procedure, do the injections with DNA buffer (10 m*M* Tris, 0.1 m*M* EDTA) alone. If the embryos survive mock injections but not injections of DNA, then the DNA should be repurified. The DNA must be free of contamination by phenol, chloroform, ethanol, etc., and the concentration should not exceed 5 μg/ml.

Another strategy to assess small litter sizes is to perform C-sections on recipient females at 19 days of gestation. When females are pregnant with one or more healthy fetuses, it will be possible to determine the total number of embryos that progressed to the implantation stage or beyond. Embryonic implantation induces proliferation of the wall of the uterus and formation of a decidium. Even if the embryo ceases development shortly after implantation, the decidium is not resorbed until after pregnancy is complete. Embryos that develop to midgestation or later can

be recognized and their stage of development estimated. Contaminants in the microinjected DNA often cause early postimplantation lethality, resulting in multiple decidua per pregnant female. Recombinant DNAs that cause dominant embryonic lethality are very rare, so the absence of transgenic mice for a specific DNA construction is typically due to some factor other than prenatal lethality of transgenic fetuses.

5. Recipient Females Do Not Become Pregnant

If the recipient females do not become pregnant, do some test embryo transfers using uninjected embryos. If pregnancies still do not occur, then embryo transfers should be practiced until the embryos can be consistently transferred to the proper region of the oviduct and the recipient females consistently become pregnant. Lack of pregnancy can also be caused by health impairment, inappropriately prepared anesthetic, or stresses in the animal housing area. Much more likely factors are poor embryo culture conditions or improper reimplantation techniques. If uninjected embryos give pregnant females, but injected embryos do not, then there is a problem with the embryo manipulations (either the protocol or the DNA).

IV. TRANSGENIC PHENOMENOLOGY

Multiple independent transgenic families have been generated in my laboratory by microinjection of the tyrosinase minigene. They provide a visual demonstration of many of the common phenomena seen in transgenic mice.

A. *Transgene Expression: Effects of Integration Site and Copy Number*

Microinjected DNA usually integrates at only one site, or a very limited number of different sites, in individual embryos. The number of copies that integrate is highly variable, ranging from just one copy to as many as 200. When multiple copies integrate, they are almost always found linked in a tandem head-to-tail array at the site of integration (Brinster *et al.*, 1981). The molecular events responsible for this pattern of integration are not well understood.

One other common observation is that there is often considerable variability in the level of transgene expression from one independent transgenic family to another. For example, with the tyrosinase minigene, pigmentation intensities have been found to range from nearly normal agouti to gray to brownish to tan to pigmented only in the ears (Figs. 8 and 9). This variability is generally attributed to

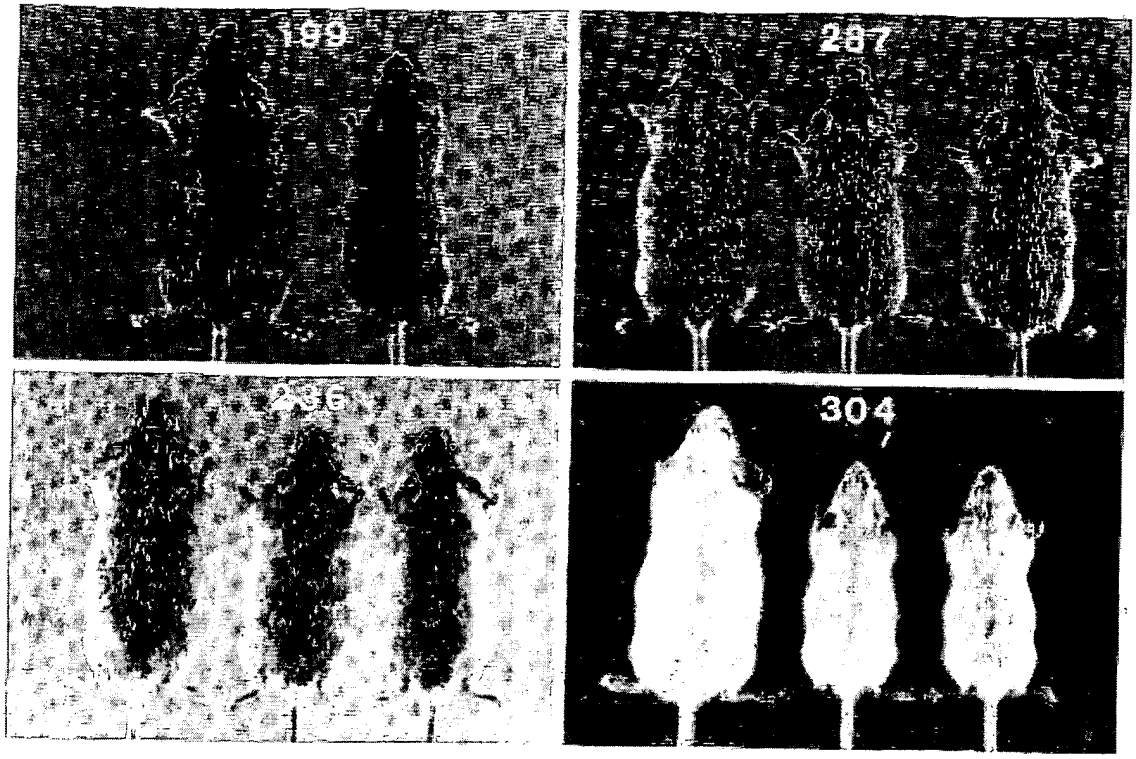

Figure 8. Variation in pigmentation. Different sites of integration of the tyrosinase minigene produce different levels of pigmentation, ranging from normal agouti (199) to gray (287) to brownish gray (736) to light tan (304). The color and intensity of pigmentation are consistent within each of the families. For each family, the mouse at left is a parent and representative pigmented offspring are shown at right.

influences of the chromosomal sequences flanking the different sites of integration. For the tyrosinase minigene there is a modest, but not consistent, correlation between the copy number of the transgene and its level of expression (see Table 3). Agouti pigmentation is present in some families that carry only two copies of the transgene, whereas other families that are pigmented only in their ears have much higher copy numbers (Table 3). The brown and tan families have consistently low copy numbers.

For some genes (such as the globins) regulatory sequences have been identified that will give levels of expression of transgenic DNA that are copy number dependent and site-of-integration independent (Grosveld *et al.*, 1987; Ryan *et al.*, 1989). These regulatory sequences often contain DNase superhypersensitive regions. Position-independent levels of expression can also be obtained by coinjection

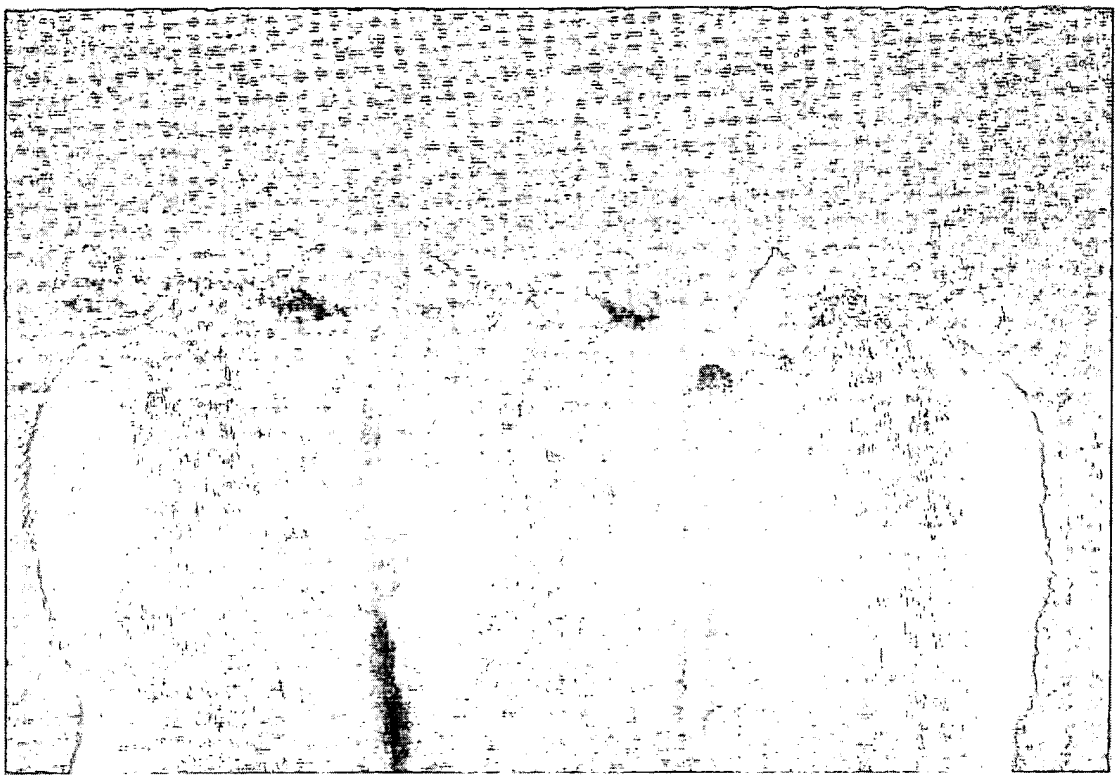

Figure 9. Pigmentation in the ears. In some of the tyrosinase transgenic families, pigmentation is readily visible only in the ears of the transgenic mice (left and center). A nontransgenic albino mouse is shown at right for comparison. (The right ear of the nontransgenic mouse has a scar where an ear tag was once located.)

of a matrix-attachment region (McKnight *et al.*, 1992). DNA purification for microinjection and removal of cloning vector sequences are discussed in Chapter 2.

B. Genetic Mosaicism in Founder Mice

DNA synthesis in mouse embryos begins 12 to 14 hr after fertilization, so that replication of the genome is generally in progress by the time that microinjections are performed. If the microinjected DNA integrates into an unreplicated location in the genome, then it can be duplicated prior to cell division, and every cell in the developing embryo will receive a copy of the integrated transgenic DNA. However, if the integration site has replicated prior to integration, then the embryo will be a genetic mosaic composed of both transgenic and nontransgenic cells. When integration occurs after just one round of DNA replication, then the founder mouse will be

TABLE 3
Comparison of Transgene Copy Number and Pigmentation Intensity for 78 Independent Integration Sites of the Tyrosinase Minigene

Pigmentation[a]	Number of families	Copy number (range)[b]	Average copy number
Agouti	14	2–40	8.0
Gray	18	2–40	7.7
Himalayan	6	4–30	16.0
Brown	13	1–8	3.8
Tan	9	1–4	2.0
Ears only	5	2–30	9.6
Dark eyes only	2	2	2.0
Mottled	5	4–16	9.6
Variable	6	4–16	10.0

[a] Pigmentation intensities were classified subjectively. The agouti mice had nearly normal pigmentation intensity, the gray mice resembled a dilute black pigmentation, himalayan mice were light gray with dark ears, brown mice showed a dilute brown phenotype, tan mice were very light brown, and certain families were pigmented exclusively in their ears or eyes. The mottled mice were partially pigmented, and the variable families showed variable intensities of pigmentation for a single integration site.

[b] Copy number was determined by Southern hybridization. The intensity of the hybridization bands for the transgene DNA was compared to the intensity for the endogenous tyrosinase gene.

approximately 50% mosaic, although the percentage may vary depending on the rates of cell division and the relative contributions of the transgenic and nontransgenic cells to the various tissues of the developing mouse.

Nonmosaic transgenic mice with one site of integration should transmit the transgenic DNA in Mendelian fashion to about 50% of their offspring, whereas mosaic mice generally show a frequency of transmission of 25% or less. (Note: Transgenic founder mice that have more than one site of integration can produce litters where 75% or more of the offspring are transgenic, although the percent transmission for any one site of integration is expected to average 50% or less. See Section IV,E for further discussion of multiple integration sites.) In some cases the transgenic DNA will integrate after the 2-cell stage, resulting in founder mice that are transgenic in substantially less than 50% of their cells. In most cases, mosaicism is recognized when the copy number of the transgenic insert is higher in the transgenic offspring than in the founder, and when the percentage transmission is substantially below 50%. With the tyrosinase minigene, mosaic mice can be identified visually, since they show a mottled pigmentation pattern (Fig. 10) that is reminiscent of chimeric mice (see Chapter 4). When the tyrosinase founder mice are mottled because of mosaicism, their transgenic offspring show a uniform pattern of pigmentation (Fig. 10). The tyrosinase minigene allows visual identification of mosaic founder mice that have only a small number of pigmented cells, and it also allows rapid identification of their rare transgenic offspring. Approximately 35% of the founder mice from our tyrosinase minigene injections were found to be genetic

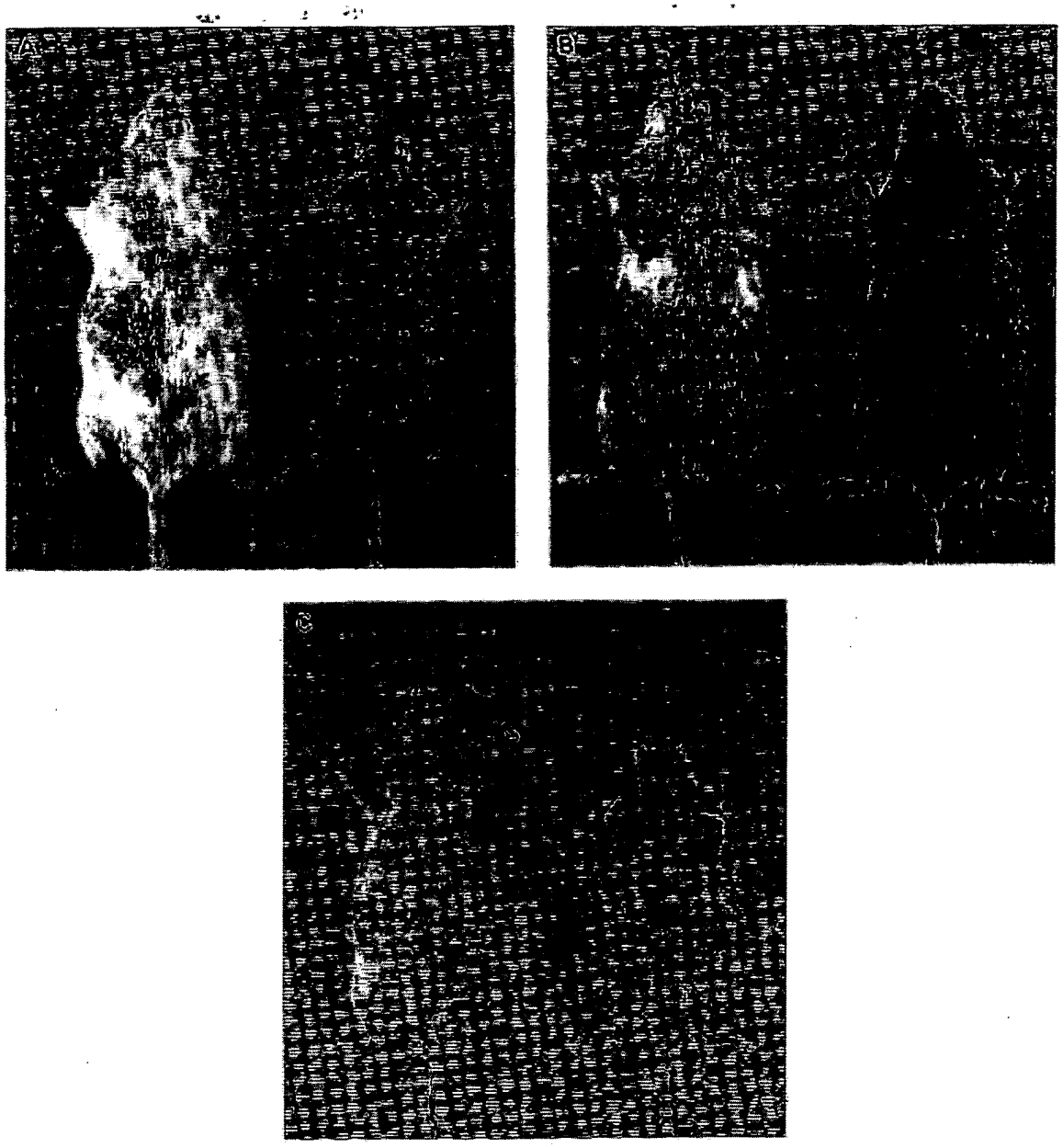

Figure 10. Mosaic founder mice. The tyrosinase minigene allows visual identification of mosaic founder mice, since the mosaic mice (at left in A, B, and C) show a heterogeneous, mottled pattern of pigmentation, whereas their transgenic offspring (at right) show uniform pigmentation.

mosaics (data not shown). For five of the mottled founder mice, the transgenic offspring still exhibited a mosaic pattern of pigmentation (e.g., Fig. 11), indicating that in these mice the mottled pigmentation was due to a mosaic pattern of transgene expression rather than to genetic mosaicism. These mice provide visual evidence that specific sites of integration can not only bias the level of transgene expression, but may also influence the cell-by-cell pattern of expression.

C. Intrafamily Variation in Expression

In some transgenic families, the level of transgene expression may vary from mouse to mouse even though the mice are inbred and have the same site of integration

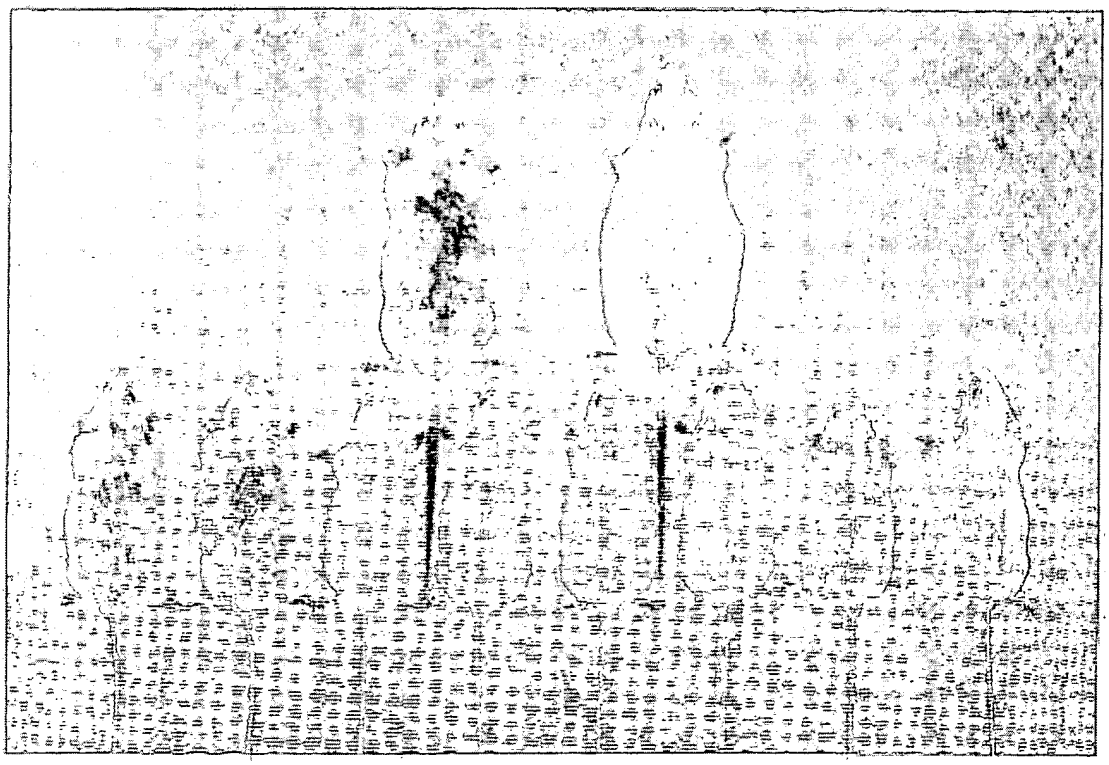

Figure 11. Mosaic expression. Some of the tyrosinase transgenic families show a persistent pattern of heterogeneous pigmentation even though the transgenic mice are genetically nonmosaic. The two mice in the top row are homozygous for the tyrosinase minigene and are the parents of the eight mice below (family CVE159). The transgenic mice in this family show variation in the intensity and specific pattern of pigmentation, but all of the mice are mottled.

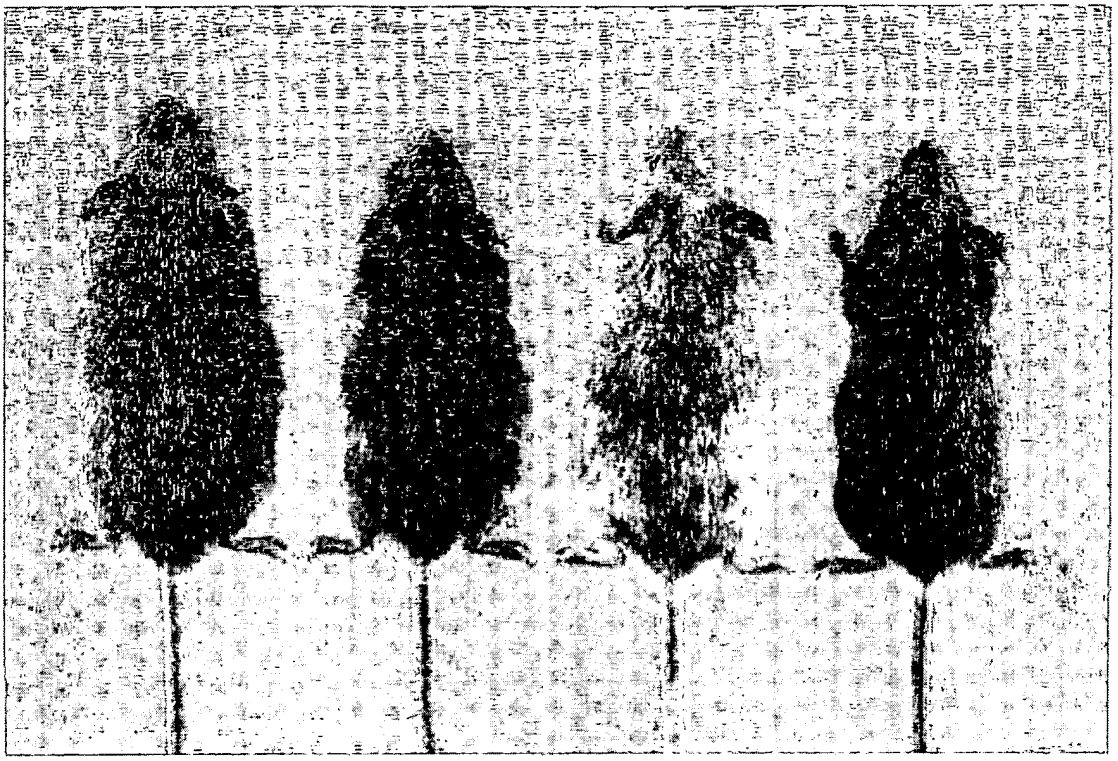

Figure 12. Inherited variability. Mice with the same site of integration can show variation in the intensity of pigmentation. In the family that is shown (OVE195), the parent at left produced the three offspring at right. Southern hybridizations showed an identical pattern of bands for all of the mice, indicating a single stable site of integration (data not shown). When the offspring mice were mated, the same variation in pigmentation was seen in the next generation. The lightly pigmented mouse had light and dark offspring, as did the more darkly pigmented mice.

(Fig. 12). In our collection of tyrosinase transgenic families, over 90% of the integration sites gave stable patterns of expression. In these cases, all of the heterozygous transgenic mice in a given family had an identical, or nearly identical, color, intensity, and pattern of pigmentation. The pigmentation did not vary from generation to generation, and the males showed the same pigmentation as the females. There were six transgenic families in which the intensity and color of pigmentation were not consistent (e.g., Fig. 12). Southern hybridizations were done to look for multiple sites of integration in these families, but the hybridization patterns were stable and consistent within each family (data not shown). The mice in these six families show an innate variability in their level of transgene expression and dem-

onstrate that mice of apparently identical genotype can exhibit variable phenotypes. The variation in expression is not correlated with the sex of the mice or of the transgenic parent, since the variability is seen between siblings.

The level of expression of transgenic DNA can also be influenced by genomic imprinting (e.g., Chaillet *et al.*, 1991; Reik *et al.*, 1990). No examples of genomic imprinting were seen in the families transgenic for the tyrosinase minigene.

D. Integration into a Sex Chromosome

Integration into either of the sex chromosomes can be identified by mating a (nonfounder) transgenic male to a nontransgenic female. If the transgenic DNA has integrated into a nonpseudoautosomal region of the X chromosome, then all the female offspring will be transgenic, whereas all the male offspring will be nontransgenic. In other words, the transgenic DNA will be transmitted along with the X chromosome to only the female offspring. The transmission pattern will be reversed for integration into the Y chromosome. Among the tyrosinase families, two integrations were found in the X chromosome and one in the Y chromosome. In the Y chromosome family, all the males and only the males were pigmented. The pigmentation was limited exclusively to the ears. In both instances where the tyrosinase minigene integrated into the X chromosome, the transgene was subject to X chromosome inactivation (e.g., Fig. 13). Heterozygous female mice showed a mottled pattern of pigmentation, whereas heterozygous males and homozygous females were uniformly pigmented.

E. Multiple Sites of Integration in Founder Mice

Founder transgenic mice occasionally have transgenic DNA integrated at more than one site in the genome. This phenomenon is typically recognized when the offspring are analyzed and found to contain two or more different copy numbers or hybridization patterns for the transgenic DNA. [Multiple sites of integration are difficult to identify by polymerase chain reaction (PCR) screening.] In general, different sites of integration are assumed to represent independent integration events. The copy numbers are variable, the levels of expression can be different, and transmission is generally random. However, transgenic families have occasionally been discovered to have different insertions in the same region of the genome (Xiang *et al.*, 1990; P. A. Overbeek, unpublished, 1990). The molecular basis for related integration events is not clear.

The tyrosinase transgenic mice allow easy identification of transgenic founder mice with multiple sites of integration, since the founder mice produce offspring

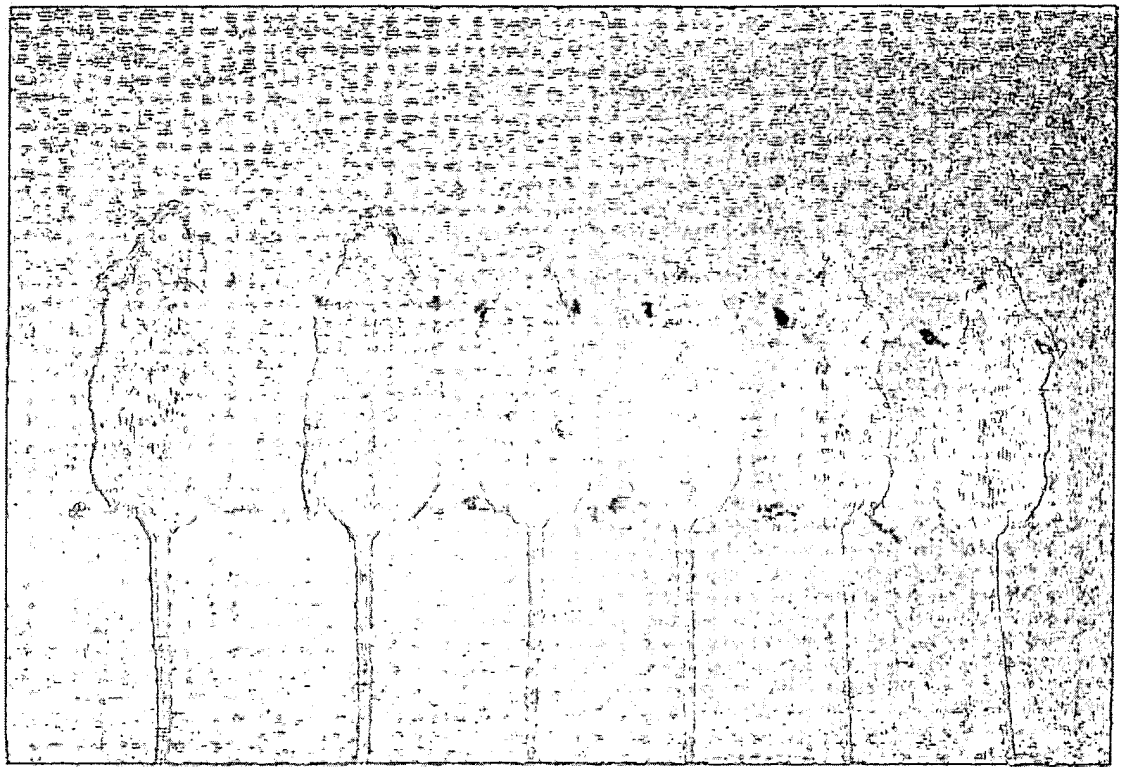

Figure 13. Inactivation of the X chromosome. A transgenic family with integration of the tyrosine minigene into the X chromosome is shown. The two mice at left are a heterozygous male and female, respectively, and are parents to the four mice at right (two males and two females). One of the females, second from the right, is homozygous for the tyrosinase minigene, whereas the other (far right) is heterozygous and mottled.

with two or more consistent colors or intensities of pigmentation (Fig. 14). Approximately 15% of the tyrosinase founder mice had either two or three different integration sites that were recognized by visual inspection of the offspring and confirmed by Southern hybridizations (not shown).

F. Homozygous versus Hemizygous

One of the major advantages of using the tyrosinase minigene to generate transgenic mice is the fact that homozygous mice in most families can be identified by simple visual inspection, since the homozygous mice have darker coat colors (Fig. 15), reflecting the increased gene dosage. In some families it has not been possible to identify homozygotes by simple visual inspection. However, homozygotes can still

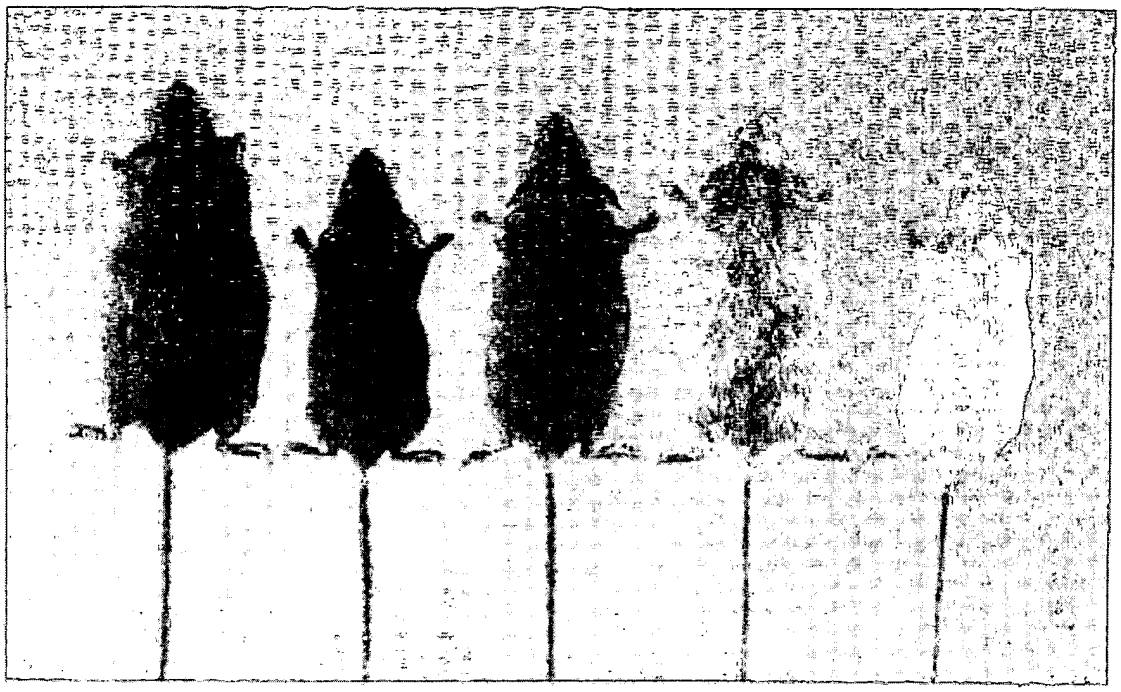

Figure 14. Founder mouse with multiple integration sites. The founder mouse for family OVE376 (at left) produced offspring with four different types of pigmentation. The middle two of the four offspring actually have the same site of integration, which is in the X chromosome. The male (left) and female (right) patterns of expression are shown.

be identified by matings to nontransgenic partners, since homozygous mice produce 100% transgenic offspring. Breeding studies to generate homozygous mice have allowed the recognition of insertional mutations that cause cleft palate (OVE270) or situs inversus (*inv* mutation, family OVE210) (Yokoyama *et al.*, 1993). In addition, five other tyrosinase families have insertional mutations that result in embryonic or neonatal lethality for the homozygous mice. These families have still been easy to maintain, since the transgenic mice in each generation can be visually identified.

G. Coinjections

The tyrosinase minigene can be coinjected with other constructs of interest. The coinjected constructs typically cointegrate into the genome, where they can be independently expressed, thereby allowing visual identification of the transgenic mice in the first and all subsequent generations (see Overbeek *et al.*, 1991).

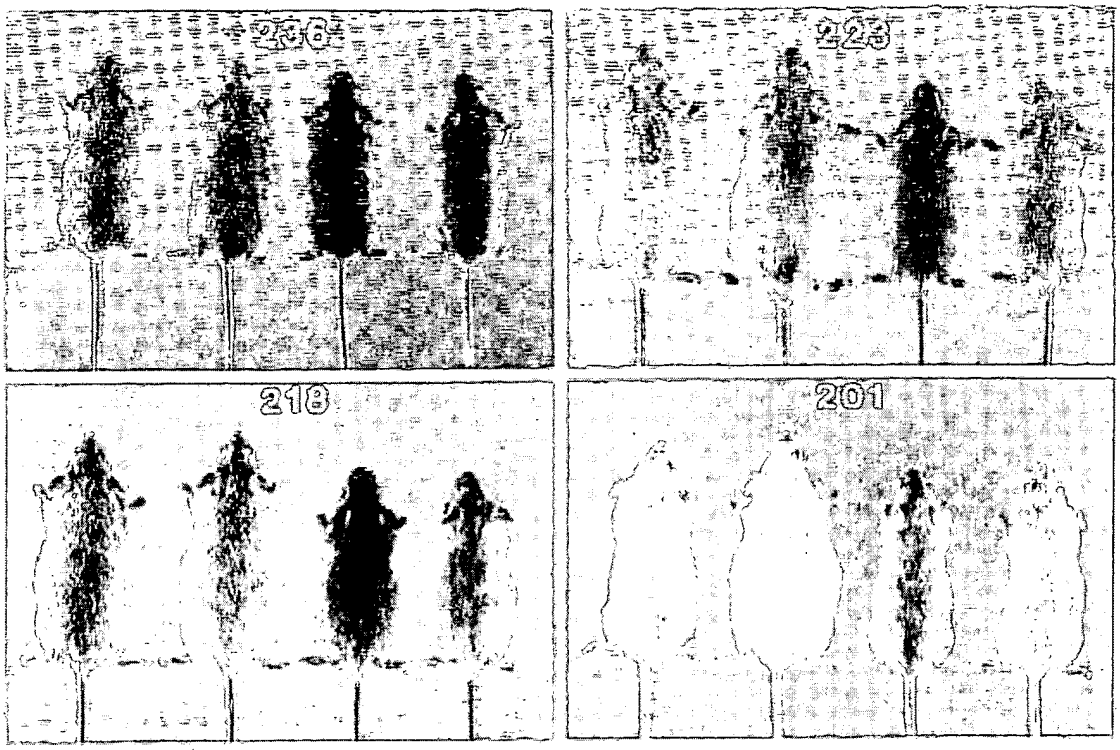

Figure 15. Visual identification of homozygotes. For most of the tyrosinase transgenic families, homozygous transgenic mice can be recognized by simple inspection for darker coat color, reflecting the 2-fold higher gene dosage. In each family shown, two heterozygous parents are shown at left, and two weanling age offspring are shown at right, one of which is homozygous (closest to the parents) while the other is heterozygous.

H. Genetic Instability

In almost all instances, transgenic DNA is stably maintained once it has integrated into the genome. Even when there are multiple copies of the DNA integrated in a tandem head-to-tail array, the transgenic DNA is transmitted stably from one generation to the next without genomic rearrangements and without deletions. However, examples of transgenic families with genetic instability under selective pressure have been identified (e.g., Sandgren *et al.*, 1991; Wilkie *et al.*, 1991). The tyrosinase transgenic families have all displayed stable Southern hybridization patterns, and those families with stable pigmentation have maintained a uniform pigmentation intensity over more than 10 generations of mating. Because alterations of the integration site would be expected to change both tyrosinase expression and

pigmentation, these results imply that genetic instability is very rare for these transgenic inserts.

V. SUMMARY

In this chapter, I have tried to provide a simplified overview of factors that often need to be taken into consideration by laboratories that are just beginning to do research with transgenic mice. I have delineated the three general strategies for generating transgenic mice and discussed the situations where each strategy is most commonly used. An introduction to different strains of mice and to husbandry techniques and troubleshooting protocols has been provided. In Section III I have reviewed some of the characteristics of transgenic mice generated by microinjection. In general, microinjected DNA appears to integrate randomly into the mouse genome. In most cases the DNA integrates stably as a tandem head-to-tail repeat containing from 1 to 200 copies of the injected DNA. Multiple factors can influence the pattern of expression of the transgenic DNA. The tyrosinase minigene can be used to allow visual identification of transgenic mice. Interestingly, certain tyrosinase transgenic mice show that, even for a stable integration at a single site in the genome, there can be variations in the level and pattern of transgene expression.

APPENDIX: STANDARDIZED NOMENCLATURE FOR TRANSGENIC ANIMALS[1]

Rules for Naming Transgenic Families

Transgenic animals should be named according to the following conventions.

Symbol. A transgene symbol consists of four parts, all in Roman type, as follows:

TgX(YYYYYY)#####Zzz

where TgX is the mode, (YYYYYY) the insert designation, ##### the laboratory-assigned number, and Zzz the laboratory code.

Mode. The first part of the symbol always consists of the letters Tg (for "transgene") and a letter designating the mode of insertion of the DNA: N for nonhomo-

1. Adapted with permission from ILAR NEWS, Volume 34, Number 4, 1992. Courtesy of the National Academy Press, Washington, D.C.

logous insertion, R for insertion via infection with a retroviral vector, and H for homologous recombination. The purpose of this designation is to identify it as a symbol for a transgene and to distinguish among three fundamentally different organizations of the introduced sequence relative to the host genome, not simply to indicate the method of insertion or nature of the vector. For example, mice derived by infection of embryos with murine leukemia virus (MuLV) vectors will be designated TgR, and mice derived by microinjection or electroporation of MuLV DNA into zygotes will be designated TgN; mice derived from ES cells by introduction of DNA followed by recombination with the homologous genomic sequence will be designated TgH, whereas mice derived by insertions of the same sequence by nonhomologous crossing-over events will be designated TgN.

Insert designation. The second part of the symbol indicates the salient features of the transgene as determined by the investigator. It is always in parentheses and consists of no more than eight characters, either letters (capitals or lowercase) or a combination of letters and numbers. Italics, superscripts, subscripts, internal spaces, and punctuation should not be used. The choice of the insert designation is up to the investigator, but the following guidelines should be used.

- Short symbols (six or fewer characters) are preferred. The total number of characters in the insert designation plus the laboratory-assigned number may not exceed 11 (see below); therefore, if seven or eight characters are used, the number of digits in the laboratory assigned number will be limited to four or three, respectively.
- The insert designation should identify the inserted sequence and indicate important features. If the insertion uses sequences from a named gene, it is preferable that the insert designation contain the standard symbol for that gene. If the gene symbol would exceed the spaces available, its beginning letters should be used. Hyphens should be omitted when normally hyphenated gene symbols are used. For example, Ins1 should be used in the symbols of transgenes that contain either coding or regulatory sequences from the mouse insulin gene (*Ins-1*) as an important part of the insert designation. Resources are available to identify standard gene symbols (see pp. 111–112). Symbols that are identical with other named genes in the same species should be avoided. For example, the use of Ins to designate "insertion" would be incorrect.
- For consistency, a series of transgenic animals produced with the same construct might be given the same insert designation. However, that is not required; some lines might manifest unique and important characteristics (e.g., insertional mutations) that would warrant a unique insert designation. If two different symbols are used for the same construct in different transgenic lines, the published descriptions should clearly identify the construct as being the same in both lines. Two different gene constructs used for transgenic animal production, either within a laboratory or in separate laboratories, should not be identified by identical insert designations. Designations can be checked through the available resources (see pp. 111–112).

• A standard abbreviation can be used as part of the insert designation (see below for an example). If a standard abbreviation is used, it should be placed at the end of the insert. These now include the following:

An	Anonymous sequence
Ge	Genomic clone
Im	Insertional mutation
Nc	Noncoding sequence
Rp	Reporter sequence
Sn	Synthetic sequence
Et	Enhancer trap construct
Pt	Promoter trap construct

This list will be expanded as needed and maintained by appropriate international nomenclature committees.

• The insert designation should identify the inserted sequence, not its location or phenotype.

Laboratory-assigned number and laboratory code. The laboratory-assigned number is a unique number that is assigned by the laboratory to each stable transmitted insertion when germ line transmission is confirmed. As many as five characters (numbers as high as 99,999) may be used; however, the total number of characters in the insert designation plus the laboratory-assigned number may not exceed 11. No two lines generated within one laboratory should have the same assigned number. Unique numbers should be given even to separate lines with the same insert integrated at different positions. The number can have some intralaboratory meaning or simply be a number in a series of transgenes produced by the laboratory. The laboratory code is uniquely assigned to each laboratory that produces transgenic animals. A laboratory that has already been assigned such a code for other genetically defined mice and rats or for DNA loci should use that code. The registry of these codes is maintained by the Institute of Laboratory Animal Resources (ILAR).

Examples. The complete designation identifies the inserted DNA, provides a symbol for ease of communication, and supplies a unique identifier to distinguish it from all other insertions. Each insertion retains the same symbol even if it is placed on a different genetic background. Specific lines of animals carrying the insertion should be additionally distinguished by a stock designator preceding the transgene symbol. In general, this designator will follow the established conventions for the naming of strains or stocks of the particular animal used. If the background is a mixture of several strains, stocks, or both, the transgene symbol should be used without a strain or stock name. The following examples are typical:

• C57BL/6J-Tg*N*(CD8Ge)23Jwg. The human *CD8* genomic clone (Ge) inserted into C57BL/6 mice from The Jackson Laboratory (J); the 23rd mouse screened in a series of microinjections in the laboratory of Jon W. Gordon (Jwg).

• Crl:ICR-TgN(SVDhfr)432Jwg. The SV40 early promoter driving a mouse dihydrofolate reductase (*Dhfr*) gene; 4 kilobase plasmid; the 32nd animal screened in the laboratory of Jon W. Gordon (Jwg). The ICR outbred mice were obtained from Charles River Laboratories (Crl).

• TgN(GPDHIm)1Bir. The human glycerol phosphate dehydrogenase (GPDH) gene inserted into zygotes retrieved from (C57BL/6J × SJL/J)F1 females; the insertion caused an insertional mutation (Im) and was the 1st transgenic mouse named by Edward H. Birkenmeier (Bir). No strain designation is provided because each zygote derived from such an F1 hybrid mouse has a different complement of alleles derived from the original inbred parental strains.

• 129/J-TgH(SV40Tk)65Rpw (hypothetical). An SV40 thymidine kinase (Tk) transgene targeted by homologous recombination to a specific but anonymous locus using embryonic stem cells derived from mouse strain 129/J. This was the 65th mouse of this series produced by Richard P. Woychik (Rpw).

Abbreviations

Transgene symbols can be abbreviated by omitting the insert. For example, the full symbol TgN(GPDHIm)1Bir would be abbreviated TgN1Bir. The full symbol should be used the first time the transgene is mentioned in a publication; thereafter, the abbreviation may be used.

Insertional Mutations and Phenotypes

The symbol should not be used to identify the specific insertional mutation or phenotype caused directly or indirectly by the transgene. If an insertional mutation produces an observable phenotype, the locus so identified must be named according to standard procedures for the species involved [Lyon, M. F. (1989). Rules and guidelines for gene nomenclature. In "*Genetic Variants and Strains of the Laboratory Mouse*" (M. F. Lyon and A. G. Searle, eds.), 2nd Ed., pp. 2–11. Oxford Univ. Press, London]. The allele of the locus identified by the insertion can then be identified by the abbreviated transgene symbol (see above) according to the conventions adopted for the species.

Examples

• $ho^{TgN(GPDHIm)1Bir}$. The insertion of a transgene into the hotfoot locus (*ho*).

• $xxx^{TgN(SVDhfr)432Jwg}$. The insertion of a transgene that leads to a recessive mutation in a previously unidentified gene. A gene symbol for *xxx* must be obtained from a species-genome database or a member of a nomenclature committee (see next section).

Resources Available for Assistance with Transgenic Nomenclature

Before naming a transgene, an investigator should obtain a laboratory code from the ILAR at the address given in the list below. An investigator who has already been assigned such a code for other genetically defined mice and rats or for DNA loci should use the same code. The transgene should be named as stated in the rules. Assistance in selecting transgene symbols is available from several organizations (see below). Lists of named genes for mice and rats are published periodically in *Mouse Genome* (Oxford University Press, Journal Subscriptions Department, Pinkhill House, Southfield Road, Eynsham, Oxford OX8 1JJ, UK) and *Rat News Letter* (Dr. Viktor Stolc, editor, *Rat News Letter*, 2542 Harlo Drive, Allison Park, Pittsburgh, PA 15101). The list of mouse genes is also maintained in GBASE, a genomic database for the mouse maintained by Dr. Don P. Doolittle, Dr. Alan L. Hillyard, Ms. Lois J. Maltais, Dr. Muriel T. Davisson, Dr. Thomas H. Roderick, and Mr. John N. Guidi at The Jackson Laboratory (see below). Human gene symbols are recorded in the Genome Data Base (GDB), which is maintained at the Johns Hopkins University (see below).

Institute of Laboratory Animal Resources (ILAR). Assigns laboratory codes; assists in naming transgenes; provides rules for naming transgenes. Contact: Dr. Dorothy D. Greenhouse, ILAR, National Research Council, 2101 Constitution Avenue, NW, Washington, DC 20418. Tel: 1-202-334-2590; Fax: 1-202-334-1687; Bitnet: DGREENHO@NAS

The Jackson Laboratory. Assists in naming transgenes; provides rules for standardized nomenclature for mice; provides lists of named mouse genes. Contact: Dr. Muriel T. Davisson, The Jackson Laboratory, Bar Harbor, ME 04609. Tel: 1-207-288-3371; Fax: 1-207-288-8982.

Medical Research Council Radiobiology Unit. Assists in naming transgenes; provides lists of named mouse genes. Contact: Dr. Josephine Peters, MRC Radiobiology Unit, Chilton, Didcot, Oxford OX11 ORD, UK. Tel: 44-235-834-393; Fax: 44-235-834-918.

Genome Data Base (GDB). Records, stores, and provides information on mapped human genes and clones. Contact: GDB, Welch Medical Library, The Johns Hopkins University, 1830 East Monument Street, Baltimore MD 21205; Tel: 1-301-955-9703; Fax: 1-301-955-0054. For assistance in naming human genes, the contact is Dr. Phyllis J. McAlpine, GDB Nomenclature Editor, University of Manitoba, Department of Human Genetics, 250 Old Basic Sciences Building, 770 Bannatyne Avenue, Winnipeg, Manitoba, Canada R3E 0W3. Tel: 1-204-788-6393; Fax: 1-204-786-8712; Bitnet: GENMAP@UOFMCC.

The Transgenic Animal Data Base (TADB). The Transgenic Animal Data Base is intended to be a comprehensive, on-line, computerized record of all lines of transgenic animals and animals with targeted mutations that have been generated worldwide. Transgenic animals of little interest to one researcher might be of enormous interest to others. This situation is addressed through the database by making available to the scientific community extensive information about transgenic constructs, including methods, expression, and phenotypes.

Scientists provide data on their own lines of transgenic animals. The TADB office faxes a set of specific questions to each scientist, who answers the questions on a floppy disk with a word-processing program. The floppy disk is returned to the TADB office, where the data are formatted and transferred to the on-line computer. The result is that pertinent information, published or unpublished, on each line of animals is organized in the database.

The TADB is accessible internationally via a toll-free number through the Tymnet telecommunications network. Users can get information at no cost from the TADB office. Contact: Ms. Karin Schneider, TADB Coordinator, Oak Ridge National Laboratory, P.O. Box 2008, MS 6050, Oak Ridge, TN 37831-6050; Tel: 1-615-574-7776; Fax: 1-615-574-9888; Bitnet: TUG@ORNLSTC; Internet: OWENSET@IRAVAX.HSR.ORNL.GOV.

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